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(54) **MUTANTS OF STREPTOKINASE AND THEIR  
COVALENTLY MODIFIED FORMS**

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Mar. 31, 2009, now Pat. No. 8,093,032.

(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**

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**C12N 9/70** (2006.01)  
**A61P 9/00** (2006.01)  
**A61K 38/48** (2006.01)  
**C07K 14/78** (2006.01)  
**C07K 14/315** (2006.01)  
**C07K 16/40** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C07K 14/78** (2013.01); **C07K 14/3153**  
(2013.01); **C07K 16/40** (2013.01); **C07K**  
**2319/00** (2013.01)

(58) **Field of Classification Search**

None  
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to novel mutants of Streptoki-  
nase, its functional fragments and covalently modified forms.  
Methods are provided for the preparation of the bacterial  
plasminogen activator protein, Streptokinase its muteins,  
species variants and their covalently modified variants that  
are characterized by improved therapeutic properties, such as  
increased proteolytic stability, extended plasma half-lives,  
reduced immuno-reactivity and enhanced fibrin clot specific-  
ity. The method involves either incorporating additional cys-  
teine residues, or substituting cysteine residues for naturally  
occurring amino acids into non-essential regions of the pro-  
tein such that the catalytic activity of the resultant protein  
remains largely unaltered. These cysteine variants were fur-  
ther modified by covalently attaching a cysteine reactive  
polymer such as polyethylene glycol (PEG) or sulfhydryl-  
reactive moieties from a group that includes fluorophore, spin  
labels or other small conjugates. Disclosed herein are site-  
specific biologically active conjugates of Streptokinases and  
its covalently modified variants.

**15 Claims, 3 Drawing Sheets**

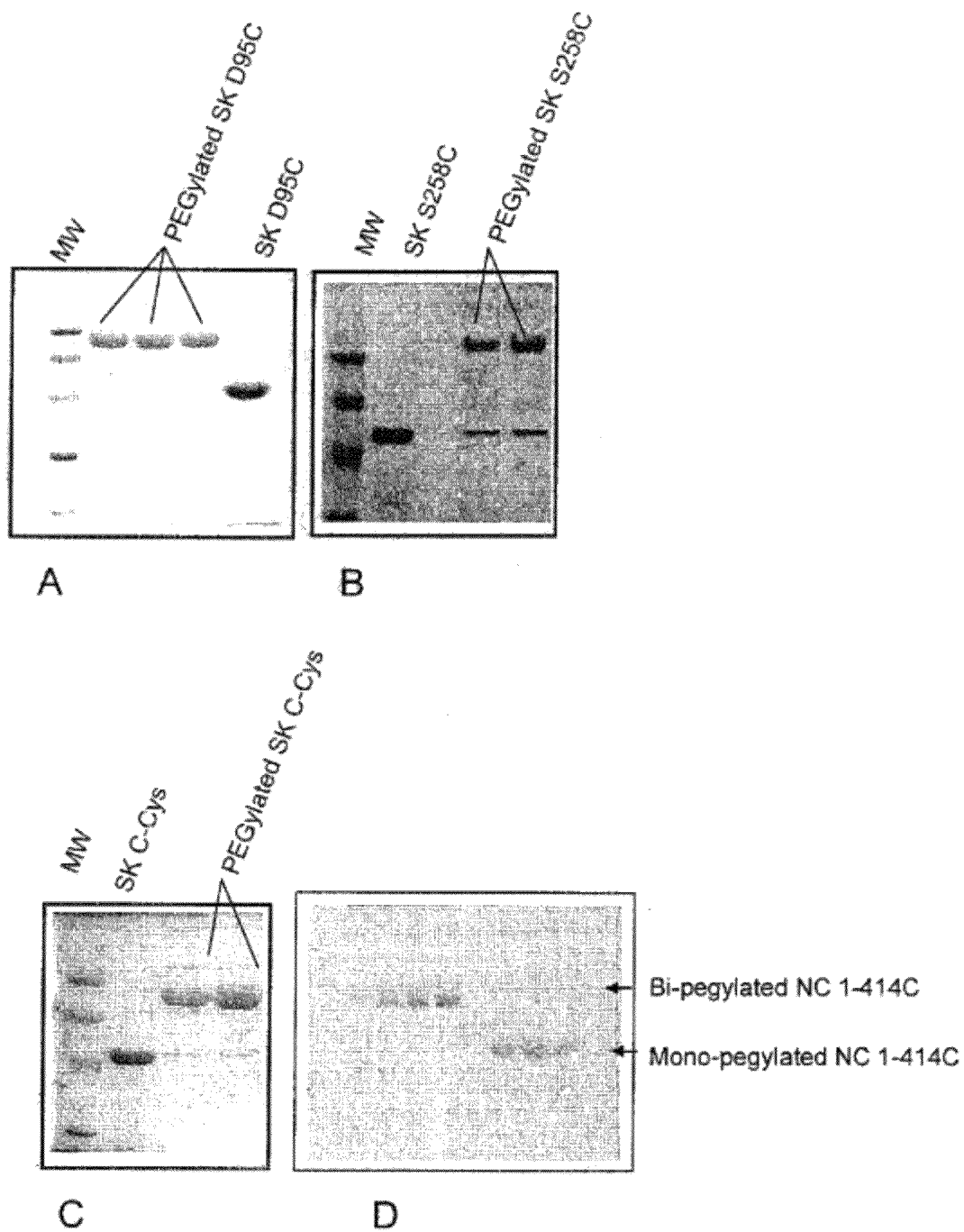


Fig.1

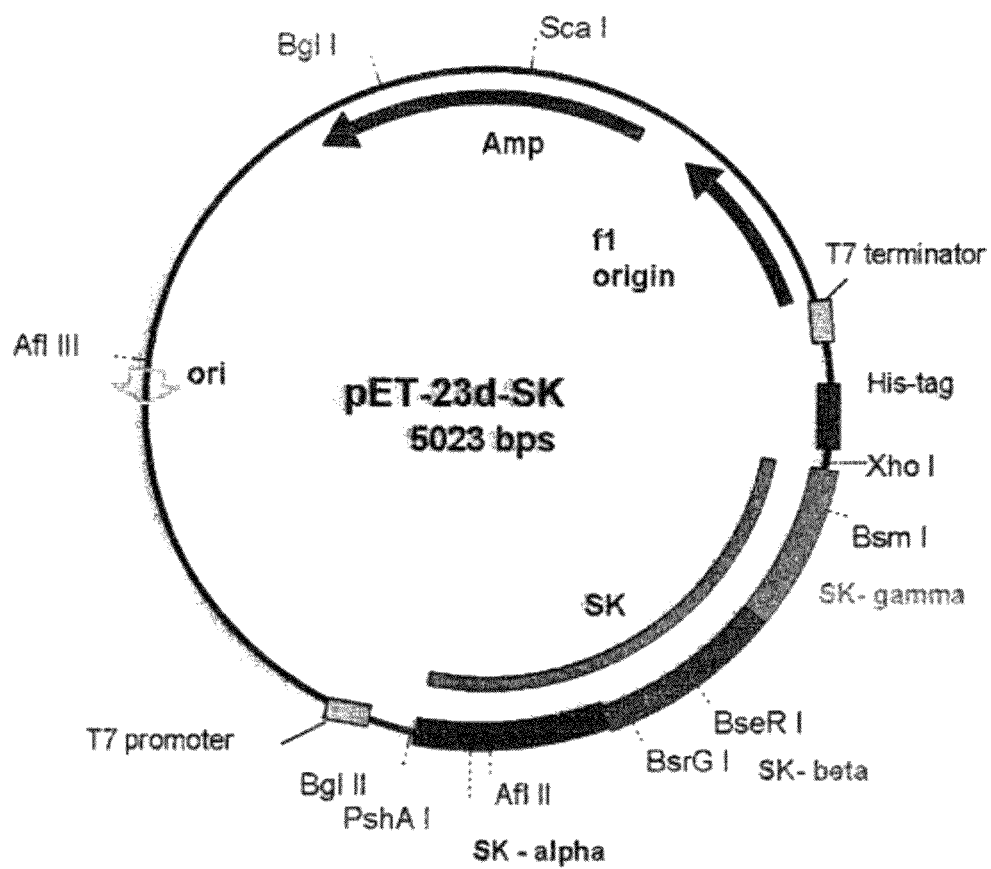


Fig. 2

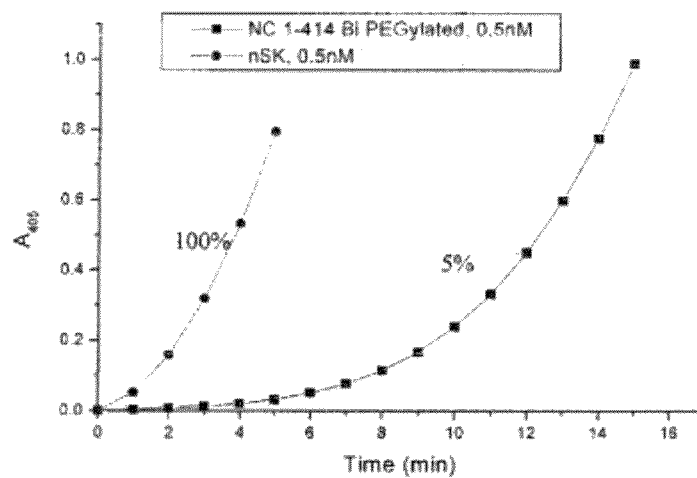


Fig. 3

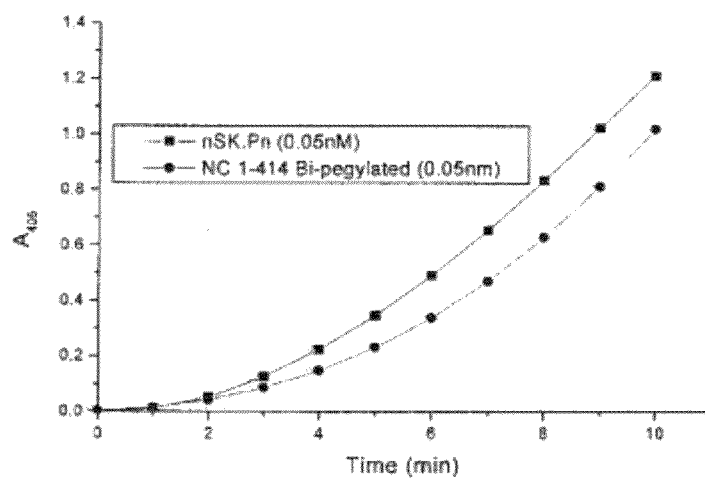


Fig. 4

## MUTANTS OF STREPTOKINASE AND THEIR COVALENTLY MODIFIED FORMS

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. patent application Ser. No. 12/415,142, filed Mar. 31, 2009, now U.S. Pat. No. 8,093,032, and Indian Application No. 0837/DEL/2008, filed 31 Mar. 2001, each of which is incorporated by reference in its entirety.

### REFERENCE TO SEQUENCE LISTING

A PDF of the sequence listing entitled "23801Z\_SequenceListing.TXT" is submitted herewith and is incorporated by reference.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to mutants of streptokinase and their covalently modified forms. The present invention utilizes the homogenous, site-specific and defined PEG modification of streptokinase and its related variants with substitutions, additions, deletions or domain fusion constructs to allow their usage in the form of improved protein therapeutics.

#### 2. Background of the Invention

Thrombus (blood clot) development in the circulatory system can cause vascular blockage leading to fatal conditions. Development of clot and its dissolution is a highly controlled process for the hemostasis. Any deviation from a normal hemostasis leads to various clinical conditions such as stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction. Patho-physiological conditions emerging out of failed hemostasis needs immediate clinical attention. The most practiced medical intervention for such cases is intravenous administration of thrombolytic agents (Collen et al., 1988; Collen, 1990; Francis and Marder, 1991). The most commonly used thrombolytic agents include Streptokinase (SK), Urokinase (UK) and the tissue type plasminogen activator (tPA). Numerous pharmacoeconomic appraisal of use of different thrombolytics in the management of acute myocardial infarction have been carried out in the past (Mucklow, 1995; Gillis and Goa, 1996). Banerjee et. al., 2004, have reviewed the clinical usefulness of streptokinase and its applicability as a drug of choice. As far as clinical efficacy is concerned both streptokinase and tPA fare equally well but due to several fold low cost and a slightly better in vivo half life, streptokinase is the most preferred thrombolytic worldwide (Sherry and Marder, 1991; Wu et al., 1998). Also, the use of tPA is slightly more likely to cause strokes, the major side effect for both the drugs. However streptokinase, being a bacterial protein is antigenic in nature and may give rise to clinical complications such as allergic response or hemorrhage. Also, the circulating half-life (15-30 min) of streptokinase is not sufficient for effective thrombolysis (Wu et al., 1998).

Despite all these, in recent years, thrombolytic therapy with fibrinolytic agents, such as Streptokinase (SK), tissue plasminogen activator (TPA) or urokinase (UK) has revolutionized the clinical management of diverse circulatory diseases e.g., deep-vein thrombosis, pulmonary embolism and myocardial infarction. These agents exert their fibrinolytic effects through activation of plasminogen (PG) in the circulation by cleavage of the scissile peptide bond between resi-

dues 561 and 562 in PG. As a result, inactive zymogen is transformed to its active form, the serine protease, plasmin (PN), which then circulates in the system and acts on fibrin to degrade the later into soluble degradation products. It may be mentioned here that PN, by itself, is incapable of activating PG to PN; this reaction is catalyzed by highly specific proteases like TPA, the SK-plasminogen complex, and UK, all of which possess an unusually narrow protein substrate preference, namely a propensity to cleave the scissile peptide bond in PG in a highly site-specific manner. However, unlike UK and TPA, SK has no proteolytic activity of its own, and it activates PG to PN "indirectly" i.e. by first forming a high-affinity equimolar complex with PG, known as the activator complex (reviewed by Castellino, F. J., 1981). The activator complex then acts as a protease that cleaves other, substrate molecules of PG to PN.

Regardless of tremendous advances in therapeutic use of streptokinase and other bacterial thrombolytics, there are several shortcomings that limit the usefulness of these polypeptide drugs. These disadvantages include their susceptibility to degradation by proteolytic enzymes, short circulating half-life, short shelf-life, rapid kidney clearance and their propensity to generate neutralizing antibodies. These shortcomings are also sometimes inherent to many other polypeptide drugs that are non human in origin. This aspect in general is reviewed by Roberts et. al; 2002. Various attempts were made to overcome these short comings in polypeptide drugs, such as altering the amino-acid sequences to reduce proteolysis or antigenicity, fusing the polypeptides to globulin or albumin domains to improve half-life etc. (Osborn et. al., 2002). These methods provided little help to the problem and came along with associated burden. The major breakthrough in this area was method of protein PEGylation that provided single solution to multiple problems. PEG (Poly Ethylene Glycol) is formed by polymerizing number of repeating subunits of ethylene glycol to give rise to linear or branched PEG polymers of tailored molecular masses. Once covalently conjugated with PEG the protein or polypeptide shows improved pharmacokinetic and pharmacodynamic properties such as increased water solubility, decreased renal clearance and often substantially limited immune reactivity (Moreadith et. al., 2003; Doherty et al., 2005; Basu et. al., 2006). The PEG conjugation also makes the molecule proteolytically less susceptible. The decreased receptor interaction or interaction with cell surface proteins that follows the PEG addition also helps to reduce adverse immunological effects. PEGylated drugs are also more stable over a wide range of pH and temperature changes (Monfardini et al. 1995). Use of PEG is FDA approved for therapeutics and it shows virtually no toxicity and eliminated from the body intact by either kidneys or in faeces. The beneficial features of PEG conjugation can be potentially imparted to SK to make it a more effective and safer thrombolytic. Attempt of SK PEGylation is reported in literature (Rajagopalan et. al., 1985) using a relatively non-specific chemical modification reaction. The therapeutic uses of such modifications were severely limited by highly compromised plasminogen activation ability. Also the nature of modification was poorly defined and heterogeneous in nature. The reason for this heterogeneity was the chemistry used for PEG modification that does not target modification of a specific site. This could be the reason why such modification strategy was not utilized for the development of improved SK based thrombolytics.

The term streptokinases used anywhere in the text collectively refers to: variants of streptokinase, any of its functional fragments, functional muteins, isolates from different species

and fusion products obtained through attachment of oligo or polypeptides of natural or artificial origin.

It is known that different functional groups present in a protein can be utilized for PEG introduction. The most commonly employed techniques are derivatization of lysine residues or cysteine residues in the protein. Alpha-amino group at the N-terminus can also be exploited for single homogenous conjugation of PEG in proteins (Baker et. al., 2006). However, the use of cysteine residues to bear the incorporated PEG groups is particularly advantageous since, potentially, the —SH groups can be targeted in a site-specific mode particularly if the protein bears or made to bear a very limited number of cysteine residues. It is not an exaggeration to state that PEG conjugation becomes an art form when the protein is devoid of any cysteine since it leaves a virtual blank canvass for cysteine addition, insertion or substitution for site-specific PEG “painting”, or decoration, of proteins. Since potentially addition of cysteines into the cysteine free background can have adverse effects on the protein function. Therefore, the selection of sites for preparation of cysteine variants requires careful planning and execution. In contrast to, say, Lysine based modifications for PEGylation, although the chemistry is well defined, heterogeneity in reaction is a big disadvantage. In the case of SK, a large number of lysine residues are evenly spread all along the polypeptide and hence limit the possibility of homogenous site-specific PEG conjugation. More interestingly, there is no natural Cysteine present in the Streptokinase molecule (Malke et. al., 1985), thus making it possible to generate various Cysteine variants of streptokinase. Also there are no free cysteines in the natively folded covalent variants of SK derived by fusion with fibrin binding domains (ref. U.S. Pat. No. 7,163,817). This renders the possibility of making various free cysteine containing variants of Clot-specific streptokinase without actually interfering with the normal refolding of the cysteine-rich protein (all the cysteine residues being engaged in disulfide bond formation). The free Cysteine(s) introduced can be reacted with various thiol-reactive reagents including PEG to generate Cysteine adduct/s of these proteins.

Streptokinase (SK) is a generic name for a secretory protein produced by a variety of hemolytic streptococci that has the ability to induce lysis of plasma clots (Tillet and Garner, 1933). Because it can be easily and economically produced from its parent source, or through rDNA technology from suitable heterologous hosts, SK is very cost effective and thus is a major thrombolytic drug particularly for the cost-conscious markets world-wide. SK has been found very effective in the clinical treatment of acute myocardial infarction following coronary thrombosis (ISIS-3, 1992) and has served as a thrombolytic agent for more than three decades. However, it suffers from a number of drawbacks. It is known that the plasmin produced through the streptokinase mediated activation of plasminogen breaks down streptokinase soon after its injection (Rajagopalan et. al., 1985; Wu et. al., 1998). This limits the in vivo half-life of streptokinase to about 15 min (Wu et. al., 1998). Although streptokinase survives in circulation significantly longer than does another thrombolytic drug of choice, TPA (with a half-life less than 5 min; Ross, 1999; Ouriel, 2002), this is still short for efficient therapy (Wu et al., 1998). Because of the recognized shortcomings related to rapid in vivo clearance of the available plasminogen activators, attempts are underway to develop improved recombinant variants of these compounds (Nicolini et al., 1992; Adams et al., 1991; Lijnen et al., 1991; Marder, 1993, and Wu et al., 1998). Despite its inherent problems, streptokinase remains the drug of choice particularly in the developing

countries because of its low relative cost (e.g., approximately US\$ 50 or less per treatment compared to nearly US \$ 1500 for TPA).

Streptokinase was first reported to cause lysis of blood clots by Tillet and Garner (1933). However, later it was established that the fibrinolytic activity of SK originates from its ability to activate human plasminogen (HPG, reviewed by Castellino, 1979). Streptokinase is mainly secreted by -hemolytic group A, C and G streptococci. SK is an activator of human PG though itself it is not a protease, rather it binds to human PG/PN and recruits other HPG molecules as substrate and converts these into product, PN. The latter circulates in the blood stream. Plasmin, being a non-specific protease, the generalized and immediate PN generation subsequent to SK injection results in large scale destruction of various blood factors leading to risk of hemorrhage, as also the dissolution of ECM and basement membrane (BM) and enhances bacterial invasiveness into secondary infection sites within the host body (Esmon and Mather, 1998; Lahteenmaki et al., 2001). Thus, there is an acute need to minimize the side-effects by designing improved SK analogs.

SK is currently being extensively used as a thrombolytic drug world wide since it is an efficient fibrin clot dissolver, yet it has its own limitations. SK being a protein produced from  $\beta$  hemolytic streptococci, its use in humans induces immunogenicity (McGrath and Patterson, 1984; McGrath et al., 1985; Schweitzer et al., 1991). The high titres of anti-SK immunoglobulins (Ig) generated after the first SK administration are known to persist in patients for several months to a few years (Lee et al., 1993). Thus, the anti-SK antibodies severely limit its use as future repeat therapy by either neutralizing SK upon administration (Spottal and Kaiser, 1974; Jalihaal and Morris, 1990) or by causing a range of allergic reactions (McGrath and Patterson, 1984; McGrath et al., 1985).

As mentioned before, the use of streptokinase in thrombolytic therapy is hampered by the relatively short half-life (a few minutes) of this protein in vivo (which indeed is the case with all presently employed thrombolytic drugs), apart from its immunogenicity. It is observed that foreign proteins when introduced into the vertebrate circulation are often cleared rapidly by the kidneys. This situation becomes even more acute in case of streptokinase where progressively higher doses of the protein (to overcome antibody based rapid neutralization) can severely increase probability of allergic response/s, making the repeated administration essentially ineffective and dangerous. Attempts to solve these problems in general, are well documented in the literature where various physical and chemical alterations have been shown to be useful for generation of improved therapeutics, e.g. see: Mateo, C. et al 2000, Lyczak, J. B. & Morrison, S. L. 1994, Syed, S. et. al; 1997, Allen, T. M. 1997. The most promising of these to-date is the approach of modification of therapeutic proteins by covalent attachment of polyalkylene oxide polymers, particularly polyethylene glycols (PEG). PEG is a non-antigenic, inert polymer and is known to increase the circulating half-life of the proteins in the body (Abuchowski et al., 1984; Hershfield, 1987; Meyers et al., 1991). This allows the extended action of the drug in use. It is believed that PEG conjugation to proteins increases their overall size and hence reduces their rapid renal clearance. PEG attachment also makes the protein or polypeptide more water soluble and increases its stability under in vivo conditions along with markedly reducing immunogenicity and increasing in vivo stability (Katre et al., 1987; Katre, 1990). U.S. Pat. No. 4,179,337 discloses the use of PEG or polypropylene glycol coupled to proteins to provide a physiologically active non-immunogenic water soluble polypeptide composition.

Although the chemistry of PEG conjugation is mostly generic but strategic placement of PEG polymers in a therapeutic protein is of paramount importance to achieve successful outcomes. Availability of three dimensional structural information with functional hot spots earmarked through various solution studies, helps in designing mutational plan to keep the functionality intact.

The complete amino acid sequence of SK was determined by sequential Edman degradation analysis of SK fragments generated by cyanogen bromide and enzymatic methods (Jackson and Tang, 1982). The results established that the molecule of Mr 47,408 Da, contains 415 amino acids in a single polypeptide chain amino acid sequence.

The nucleotide sequence from *S. equisimilis* H46A (the prototype strain for SK production that is most often used therapeutically in humans) was sequenced by Malke and co-workers, in 1985. The transcriptional control of this gene has also been studied and the functional analysis of its complex promoter has been reported (Grafe et al., 1996). Considerable information exists, therefore, for effectively using this gene in producing streptokinase safely in relatively non-pathogenic microbes.

#### SUMMARY OF THE INVENTION

Accordingly, the present invention provides mutants of streptokinase, its functional fragments or covalently modified forms. The variants comprise polypeptides related to SK where one or more Cysteine residues are substituted for one or more non-essential amino-acids of the proteins. Preferably the variants comprise a Cysteine residue substituted for an amino-acid selected from amino-acids in the loop regions, the ends of the alpha helices and even in the secondary structure-forming regions, or regions wherein the Cysteine residue is added at the N-terminus or C-terminus of the proteins with or without added amino acid extensions.

The present invention involves the general methods for the selection, production and use of streptokinases that show increased proteolytic stability, extended plasma elimination half-life and reduced immunogenicity. The derivatives have modified amino-acid sequences but retain their biological activity effectively. The invention also describes cysteine variants of Streptokinases that are covalently attached to one or more molecules of polyethylene glycol (PEG) of various molecular weights such as at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 45, 50, 55, 60, 65 or 70 kDa, or more. Of course, any of the indicated weights above can serve as a lower and upper limit to a range of molecular weights. For example, the PEG used in the present invention may have a molecular weight of between about 1 kDa and about 2 kDa, or between about 1 kDa and about 3 kDa, or between about 2 kDa and about 6 kDa, etc. One of the embodiments of the present invention encompasses pharmaceutical compositions of the PEGylated Streptokinase derivatives together with suitable excipients, stabilizers, and carriers as are known in the art for effective dissemination in the body for the treatment of diverse circulatory disorders.

The present invention relates to covalent attachment of PEG to cysteine variants of streptokinase, its muteins, species variants or fibrin fusion products, using thiol reactive PEG reagents. One can also use the different pKa value of alpha amine groups to carry out alpha-amine specific PEG conjugation at acidic pH to generate mono-PEGylated derivatives of streptokinase or its muteins.

The present invention also relates to identifying various Cysteine variants of Streptokinase, or its mutants including

related covalent variants on the basis of structural and functional information (Wang et al., 1998). Structural comparison of other one domain plasminogen activator, staphylokinase (SAK) shows strong similarity with SK alpha domain although the two have no significant sequence homology at amino acid level (Rabijns et al., 1997). This indicates that plasminogen activators from different sources retain the same structural fold even if they differ much in their polypeptide sequence. The evolutionary constrain to keep the structural integrity is expected because bacterial plasminogen activators are protein cofactors, therefore utilize multiple contact points required for conformational activation of zymogen. Such structural similarity extends the scope of this invention further because the methods practiced in this case can be applied to bacterial plasminogen activators from other genre or species that share the similar plasminogen activation domains. For this reason, the "rules" disclosed herein for creating biologically active cysteine variants of streptokinase will be useful for creating biologically active cysteine variants of various other forms of streptokinase. Conjugation of these cysteine variants with cystine-reactive PEGs will impart similar benefits as obtained with the variants used in this study. The rules for determining the site of Cysteine placement was largely based on the idea to choose from the surface exposed residues falling either in the loop or helix or the sheets or in the boundaries of structural and flexible regions. To determine the surface accessibility DSSP program was used. The DSSP (Kabsch et al., 1983) program defines secondary structure, geometrical features and solvent exposure of proteins, given atomic coordinates in Protein Data Bank format. DSSP states each residue's exposure in terms of square Angstroms. Surface accessibility of the streptokinase amino-acid residues were deciphered from high resolution crystal structure of streptokinase in complex of microplasmin (Wang et al., 1998, PDB ID IBML). For the regions that were missing in this structure (175-181 and 252-262) crystal structure of the isolated beta domain (Wang et al., 1999, PDB ID 1c4p) was used for determination of surface exposure.

Cysteine variants of streptokinase its muteins, species variants and its covalently modified forms were further chemically modified by attaching sulfhydryl reactive reagents, followed by empirical testing for substantial retention of biological activity along with gain in new properties such as reduced immunogenicity, or reaction with anti-SK antibodies, reduced proteolytic susceptibility, increased in vivo survival etc. More particularly, the invention relates to production of engineered streptokinase derivatives for use in pharmaceutical compositions for treating circulatory disorders.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts an SDS-PAGE showing purified cysteine variants of streptokinase and their PEG adducts. Panel A shows cysteine variant of alpha domain (SEQ ID NO: 30) of streptokinase where aspartate present at 95th position in the flexible loop 88-97 has been replaced with cysteine. This unique cysteine so generated has been PEGylated with thiol reactive PEG polymer methoxy-PEG maleimide of 20 KDa followed by purification to give a homogeneous protein-PEG adduct. Panel B shows cysteine variant of the SK beta domain (SEQ ID NO: 49) where serine at 258th position in the 250 loop of beta domain has been replaced with cysteine. This unique cysteine so generated has been PEGylated with thiol reactive PEG polymer methoxy-PEG maleimide to give protein-PEG adduct of higher molecular weight. Panel C shows cysteine variant of streptokinase where the original sequence

of streptokinase has been extended by one more amino-acid by adding cysteine at C-terminus position (SEQ ID NO 490). The thiol so generated at C-termini (C-CYS) has been cross-linked with methoxy-PEG maleimide of 20 KDa to give protein-PEG adduct of higher molecular weight. Panel D shows the bi-pegylated variant of streptokinase (SEQ ID NO: 491), where cysteine has been placed one each at both N and C-terminus of the original sequence of streptokinase. The double cysteine mutant so generated has been modified with PEG of molecular weight 20 KDa.

FIG. 2 depicts the circular map of pET-23d-SK, the expression vector employed for the expression of streptokinase in *E. coli*. The circular map highlights a few selected, unique RE sites on the pET-23d vector, a T7-RNA polymerase promoter-based expression vector (Studier et al., 1990) and the incorporated gene encoding for SK that was used for the construction and expression of SK and its muteins.

FIG. 3 shows the spectroscopically obtained progress curves of HPG activation by nSK and bi-pegylated streptokinase variant NC 1-414 (SEQ ID NO: 491). For reaction 0.5 nanomolar of nsk abd bi-pegylated NC 1-414 were added to two different wells of multiwall plate already containing 1 micromole of HPG and 0.5 mM of chromogenic substrate (S-2251). The reaction was then monitored spectrophotometrically at 405 nm. The circles show the progress curves for nSK while squares denote progress curve for bi-pegylated NC 1-414. Noticeably, the progress curves show significant lag in the ability to activate plasminogen for bi-pegylated NC 1-414 but not in case of nSK.

FIG. 4 shows progress curves of HPG activation by equimolar complex of nSK or bi-PEGylated NC 1-414 (SEQ ID NO: 491) with human plasmin. Catalytic amount (0.05 nm) of either equimolar complexes were added to multiwall plate already containing 1  $\mu$ M of HPG and 0.5 mM of chromogenic substrate (S-2251). Progress curves show the comparable plasminogen activability for both nSK and the bi-pegylated SK variant (NC 1-414). This proves the notion that physical capping of SK at termini generates a "Plasmin Switch" in the molecule and restricts or delay its plasminogen activation capability in absence of plasmin. The use of pre-formed complex of bi-PEGylated streptokinase NC 1-414 with plasmin abolishes the time lag seen in FIG. 3, and thus establishes the plasmin dependency for normal plasminogen activation ability by the PEGylated SK which is in contrast to nSK which does not show a lag irrespective of the absence/

#### DETAILED DESCRIPTION OF THE INVENTION

The main object of the present invention is to provide mutants of streptokinase with potential for increased efficacy due to extended action and reduced immuno-reactivity.

Another object of the present invention relates to novel mutants of Streptokinase, its functional fragments and covalently modified forms.

Another object of the invention is to provide methods for the preparation of the bacterial plasminogen activator protein, Streptokinase its muteins, species variants and their covalently modified variants that are characterized by improved therapeutic properties, such as increased proteolytic stability, extended plasma half-lives, reduced immune-reactivity and enhanced fibrin clot specificity. The methods involve either incorporation additional cysteine residues, or substituting cysteine residues for naturally occurring amino acids into non-essential regions of the protein such that the catalytic activity of the resultant protein remains largely unaltered.

Yet another object of the present invention is to provide a method for the production of PEGylated cysteine variants of streptokinase or its active muteins or the hybrid plasminogen activator molecules in pure and biologically active form.

Yet another object of the present invention is to provide a mutant streptokinase polypeptide comprising from one to three cysteine substitutions, wherein the cysteine substitution is located acid in at least one region corresponding to the native amino acid sequence of Streptokinase (SEQ ID NO: 1), the region being selected from the group consisting of the loop of amino acid residues 48-64, the loop of amino acid residues 88-97, the region of amino acid residues 102-106, the region of amino acid residues 119-124, the helix forming region of amino acid residues 196-207, the loop forming region of amino acid residues 170-181, the loop forming region of amino acid residues 254-264, the coiled coil region of amino acid residues 318-347 and the region of amino acid residues 360-372, wherein the mutant can activate plasminogen.

In another embodiment the mutant streptokinase comprises at least one amino acid substitution, the amino acid substitution corresponding to an amino acid substitution being selected from the group consisting of Asn90Ala, His107Ala, Ser108Ala, Asp227Tyr, Asp238Ala, Glu240Ala, Arg244Ala, Lys246Ala, Leu260Ala, Lys278Ala, Lys279Ala and Asp359Arg of SEQ ID NO:1.

In another embodiment the mutant streptokinase comprises at least one cysteine mutation at a position corresponding to G49, S57, A64, 188, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360 or R372 of SEQ ID NO:1.

In another embodiment the mutant streptokinase comprises at least two cysteine mutations at a position corresponding to G49, S57, A64, 188, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360 or R372 of SEQ ID NO:1.

In another embodiment the mutant streptokinase comprises at least three cysteine mutations at a position corresponding to G49, S57, A64, 188, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360 or R372 of SEQ ID NO:1.

In another embodiment the mutants, further comprise a fibrin binding domain fused to the C-terminus, the N-terminus or both termini. In another embodiment, the fibrin binding domain is connected to the mutant streptokinase via a flexible connecting oligopeptide.

In another embodiment, the mutants comprising a fibrin-binding domain comprise at least one cysteine substitution in the fibrin binding domain.

In another embodiment the mutant streptokinase comprises a deletion, the deletion corresponding to an amino acid deletion being selected from the group consisting of Asn90, Asp227 and Asp359 of SEQ ID NO:1.

Yet another object of the present invention is to provide a fusion polypeptide, the fusion polypeptide comprising a streptokinase domain and a fibrin binding domain, the streptokinase domain comprising from one to three cysteine substitutions, wherein the cysteine substitution is located in the fibrin binding domain or at least one region corresponding to the native amino acid sequence of Streptokinase (SEQ ID NO: 1), the region being selected from the group consisting of



the loop of amino acid residue 48-64, the loop of amino acid residues 88-97, the region of amino acid residues 102-106, the region of amino acid residues 119-124, the helix forming region of amino acid residues 196-207, the loop forming region of amino acid residues 170-181, the loop forming region of amino acid residues 254-264, the coiled coil region of amino acid residues 318-347, and the region of amino acid residues 360-372 of SEQ ID NO: 1, wherein the mutant can activate plasminogen and bind fibrin.

In another embodiment, the mutant streptokinase comprises at least one cysteine mutation selected from the group consisting of H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477 and R489 of SEQ ID NO:22.

In another embodiment, the mutant streptokinase comprises at least one cysteine mutation selected from the group consisting of G49, S57, A64, I88, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360, R372, H401, A402, D447 and G465 of SEQ ID NO:23.

In another embodiment, the mutant streptokinase comprises at least one cysteine mutation selected from the group consisting of H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477, R489, H518, A519, D564 and G582 of SEQ ID NO:24.

In another embodiment the mutant streptokinases further comprise an N and/or C-terminus extension of amino acids.

In another embodiment the mutant streptokinase can be used in conditions associated with thrombosis. For example, the mutant streptokinases can be used to treat a disease or disorder selected from the group consisting of myocardial infarction, vascular thromboses, pulmonary embolism, stroke, a vascular event, angina, pulmonary embolism, transient ischemic attack, deep vein thrombosis, thrombotic re-occlusion subsequent to a coronary intervention procedure, peripheral vascular thrombosis, heart surgery, vascular surgery, heart failure, Syndrome X and a disorder in which a narrowing of at least one coronary artery occurs.

In another embodiment the mutant streptokinases further comprise a cysteine-reactive moiety substituted on at least one of the cysteine mutants. In another embodiment the cysteine-reactive moiety is polyethylene glycol (PEG). In another embodiment the PEG is a linear or branch polymer of molecular size ranging from 5000 daltons-40,000 daltons.

In another embodiment the mutant streptokinases comprising PEG have increased proteolytic stability, compared to an un-PEGylated mutant streptokinase. In another embodiment, the mutant streptokinases comprising PEG have decreased antigenicity and decreased in vivo immunogenicity, compared to an un-PEGylated mutant streptokinase. In another embodiment the mutant streptokinases comprising PEG have slow renal clearance and increased in vivo half life, compared to an un-PEGylated mutant streptokinase.

The present invention is based on the experimental findings that covalent attachment of one or more molecules of PEG to the strategically substituted or added cysteine residues in the streptokinase results in a biologically active, PEGylated streptokinase with increased proteolytic stability and extended elimination half-life along with reduced clearance, and lesser immune reactivity when compared to native strep-

tokinase. The site and size of PEG conjugation can be tailor-made with the help of cysteine variants designed to be non-inhibitory to catalytic function (plasminogen activation) in the streptokinase and its active variants, including clot-specific streptokinases (reference in this context may be made to U.S. Pat. No. 7,163,817 which describes the design and construction of clot-specific streptokinase variants with increased fibrin affinity due to the addition of fibrin binding domains to either, or both, ends of the SK protein). Substitution, addition or insertion of one or more cysteine/s in the streptokinase and clot-specific streptokinase polypeptides makes it convenient to add PEG of different molecular masses to the desired location of the polypeptide provided the substitutions and or additions are carried out in a "strategic" manner that cleanly avoids loss of functional characteristics, and result in the generation of new beneficial properties not present in the unmodified native protein. The choice of PEG placement was designed on the basis of surface accessibility of the selected site and its structure-function relevance.

The PEGylated streptokinases of the present invention have greater usefulness as therapeutics as well as greater convenience of use compared to the native molecule(s) because while retaining native or near-native like biological activity, they exhibit an extended time-action when compared to the former, which degrade rapidly in vitro in the presence of plasmin(ogen), as well as after injection as a result of which these are cleared from the circulation in a very short time. In contrast, the PEGylated streptokinases exhibit significantly enhanced proteolytic stability, are less immuno-reactive, and are cleared from the circulation in vivo only after markedly extended durations as compared to the native (nonPEGylated) proteins.

Therefore, PEGylated streptokinases of the present invention are useful to treat subjects with circulatory disorders such as venous or arterial thromboses, myocardial infarction etc, with the advantages being that the PEGylated streptokinases of the invention present the potential for increased efficacy due to extended action and reduced immuno-reactivity, that allows the possibility of repeated administration due to their minimal antigenicity. The number, size and location of PEG group/s can be employed in such a way so as to redesign the Streptokinases to possess differential half-lives so to make their use conducive to the requirement of a particular disorder/clinical syndrome, or a particular subject under treatment.

The present invention involves the selective modification of streptokinases for pharmaceutical use, to both enhance its pharmacokinetic properties and provide therapeutically useful thrombolytics. This invention also include mutants of streptokinase its natural or artificial variants that retain desirable biological properties of the native unmodified molecule. All variants of this invention may be prepared by expressing recombinant DNA sequences encoding the desired variant in host cells, e.g. prokaryotic host cells such as *E. coli*, or eukaryotic host cells such as yeast or mammalian cells, using conventionally used methods and materials known in the art. DNA sequence information for encoded streptokinase from different species may be obtained from published information. Polymorphism of the streptokinase gene has been studied and their implications for the pathogenesis are explained (Malke H, 1993). A molecular epidemiological study has also been conducted to determine the distribution of the streptokinase gene in group A streptococcal strains of different M types and in other streptococcal species. Most of the strains examined in this study show positive streptokinase activity by the casein-plasminogen overlay assay. The overall results of these studies indicate that there is considerable heterogeneity among the streptokinases obtained from different streptococ-

cal species (Huang et. al; 1989). It is possible to use any of available streptokinase variant that has plasminogen activation ability for cysteine mutagenesis and subsequent modifications with sulfhydryl reactive agents.

The new DNA sequences encoding mutants and species variants can be similarly cloned and expressed as in case of natural forms. The streptokinases produced by expression in the genetically engineered host cells may then be purified, and if desired formulated into pharmaceutical compositions by conventional methods.

As a preferred aspect of this invention, the streptokinases expressed by recombinant means are reacted with the desired thiol reactive agents under conditions that allow attachment of the thiol reactive moiety to the sulfhydryl group of the introduced cysteine residues in the streptokinases.

The term thiol reactive is defined herein as any compound having, or capable of being activated to have, a reactive group capable of forming a covalent attachment to the sulfhydryl group (—SH) of the cysteine residue. Included among such compounds are polymers such as polypropylene glycol and PEG, carbohydrate based polymers and polymers of amino-acids and biotin derivatives. Compound need to be conjugated can be activated with a sulfhydryl moiety, such as sulfhydryl group, thiol, triflate, tresylate, aziridine or oxiran, or preferably, iodoacetamide or maleimide. The conjugating group may have various molecular weights but preferably between 5000 and 40,000 for the PEG. One of the important attributes of the present invention is to confer positional selectivity of the PEGylation or other attachments while preserving the normal functional properties of the protein.

Accordingly, the present invention provides a mutant streptokinase polypeptide having amino acid sequence selected from the group consisting of SEQ ID NO: 1-24, wherein at least one cysteine residue is substituted or inserted. Table 34 shows residues that correspond to residues of SEQ ID NO:1 that are likely intolerant to mutation or substitution.

In an embodiment of the present invention, the mutant of streptokinase prepared is a functional fragment of streptokinase having SEQ ID NO: 2-6.

In an embodiment of the present invention, the mutants of streptokinase prepared are muteins of streptokinase having SEQ ID NO: 7-19.

In an embodiment of the present invention, the mutant of streptokinase prepared are species variants of streptokinase having SEQ ID NO: 20-21.

In an embodiment of the present invention, the species variants of streptokinase show 75%-100% amino acid sequence homology with the native streptokinase having SEQ ID NO: 1.

In another embodiment of the present invention, at least one cysteine residue is substituted for at least one amino acid located in at least one region of Streptokinase selected from the group consisting of: the 48-64 loop, 88-97 loop, the region 103-106, or 119-124 or the helix forming region 196-207 or the loop forming region 170-181 or the loop forming region 254-264 or the coiled coil region 318-347 or the region 360-372 of SEQ ID NO: 1 or its muteins or their functional fragments, wherein said variant has biological activity as measured by a standard assay.

As used herein, the term corresponding to is used to mean enumerated positions within the reference protein, e.g., wild-type Streptokinase (SK) or SEQ ID NO:1, and those positions in the queried protein (e.g. a mutant SK) that align with the positions on the reference protein. Thus, when the amino acid sequence of a subject SK, e.g., SEQ ID NOs: 2, 3, 4, 5, etc., is aligned with the amino acid sequence of a reference SK, e.g., SEQ ID NO:1, the amino acids in the subject SK sequence

that “corresponds to” certain enumerated positions of the reference SK sequence are those that align with these positions of the reference SK sequence, but are not necessarily in these exact numerical positions of the reference SK sequence. For example, a Gly34Cys mutant in SEQ ID NO:4 would “correspond to” a Gly49Cys mutant in SEQ ID NO:1.

In yet another embodiment of the present invention SEQ ID NO: 22-24 are covalently modified hybrid polypeptide comprising of at least one functional fragment of streptokinase (SK) and fibrin binding domains 4 and 5, fibrin binding domains (FBDs) 1 and 2 of human fibronectin.

In yet another embodiment of the present invention, the functional fragment of SK and said fibrin binding domains are connected via a flexible connecting oligopeptide.

In yet another embodiment of the present invention, the mutant described above comprises an N and/or C-terminus extension of amino acids.

In yet another embodiment of the present invention, a cysteine residue is substituted for at least an amino acid selected from the group consisting of: G49, S57, A64, I88, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360, R372, wherein said variant has biological activity as measured by a standard assay.

In yet another embodiment of the present invention, a cysteine residue is substituted for at least an amino acid selected from the group consisting of: H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477, R489, of the SEQ ID NO. 22, wherein said variant has biological activity as measured by a standard assay.

In yet another embodiment of the present invention, a cysteine residue is substituted for at least an amino acid selected from the group consisting of: G49, S57, A64, I88, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205, A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360, R372, H401, A402, D447, G465, of the SEQ ID NO. 23, wherein said variant has biological activity as measured by a standard assay.

In yet another embodiment of the present invention, a cysteine residue is substituted for at least an amino acid selected from the group consisting of: H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477, R489, H518, A519, D564, G582 of the SEQ ID NO. 24 which has biological activity as measured by a standard assay.

In yet another embodiment of the present invention, the substituted cysteine residue is modified with a cysteine-reactive moiety.

In yet another embodiment of the present invention, substituted cysteine residue is modified with polyethylene glycol.

In yet another embodiment of the present invention, the PEG molecule stated above is a linear or branch polymer of molecular size ranging from 5000 daltons-40,000 daltons.

In yet another embodiment of the present invention, the variant described above has increased proteolytic stability as compared to their original unmodified counterparts.

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In yet another embodiment of the present invention, the above described variant has decreased antigenicity and in vivo immunogenicity when compared to their original unmodified counterparts.

In yet another embodiment of the present invention, the above described variant has slow renal clearance hence increased in vivo half life as compared to their original unmodified counterparts.

In yet another embodiment of the present invention, the pharmaceutical composition comprises at least one of the cysteine variants optionally along with pharmaceutically acceptable excipient(s).

In yet another embodiment of the present invention, the pharmaceutical composition is useful for treating disease or disorder selected from the group consisting of myocardial infarction, vascular thromboses, pulmonary embolism, stroke a vascular event, angina, pulmonary embolism, transient ischemic attack, deep vein thrombosis, thrombotic reocclusion subsequent to a coronary intervention procedure, peripheral vascular thrombosis, heart surgery or vascular surgery, heart failure, Syndrome X and a disorder in which a narrowing of at least one coronary artery occurs.

Particularly the present invention features PEGylated cysteine variants of streptokinase or its muteins, or of a hybrid plasminogen activator comprising a polypeptide bond union between streptokinase (SK), or modified forms of SK, or suitable parts thereof, which are capable of plasminogen (PG) activation, with fibrin binding regions of human fibrinectin selected from the fibrin binding domains of human fibrinectin (e.g. the pair of domains 4 and 5, or domains 1 and 2, or modified forms thereof), so that the hybrid plasminogen activators possess the ability to bind with fibrin independently and thereby become clot specific due to their enhanced affinity for the substance of the blood clot, namely fibrin (U.S. Pat. No. 7,163,817).

It provides mono- or bi- or multi-PEGylated Cysteine variant/s of streptokinase or its truncated forms that are not only active with respect to PG activation capability, but exhibit a new and unexpected functional attribute. For example, the bi-PEGylated cysteine variant of SK where additional cysteines are placed at the two extremities of the polypeptide i.e. at the N- and C-termini, exhibits an unexpected property in respect to its human plasminogen activation characteristics, in that it has a markedly slower initial rate of activation of plasminogen (PG) compared to unmodified SK, but becomes fully capable of activating plasminogen in a manner similar to that of unmodified SK after an initial lag of several minutes' duration when assayed for PG activation in vitro. The inability to be self-activated immediately (as is the case with native, unmodified SK which activates PG virtually upon contact) is due to a plasmin-dependent mode of its action. In contrast, native SK does not require any plasmin to be activated, but is activated virtually as soon as it complexes with PG. Thus, after injection into the body, such a SK variant will make its voyage through the vascular system while still in an inactive, or partially active, state. However, it will preferentially become activated in the immediate vicinity of the clot the moment it contacts the clot, which is known to be plasmin-rich whereas the general circulation is not (free plasmin being rapidly inactivated in the 'open' circulation due to the presence of plasmin-specific Serpins [serine protease inhibitors] such as alpha-2-antiplasmin and alpha-2-macroglobulin), thereby obviating or significantly minimizing the systemic PG activation coincident with natural SK administration which immediately activates PG upon administration with consequent side-effects such as hemorrhage and large scale destruction of various protein components of the vascular

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system. This property i.e. plasmin-dependant activation, along with the extended elimination half-life, and low immunogenic and antigenic reactivity would result in not only an overall diminished generation of free plasmin in the general circulation but also the ability for the thrombolytic agent to be administered repeatedly for various circulatory maladies in a relatively lower dose while avoiding unwanted immune reactions. The net result shall be a continued and more efficient fibrinolysis at the target sustained by considerably lowered therapeutically effective dosages of the thrombolytic agent with minimized side-effects such as lowered immune reactivity, and mitigation of hemorrhagic complications often seen with normal SK.

The invention provides PEGylated cysteine variants of streptokinase or its muteins or of a hybrid plasminogen activator comprising a polypeptide bond union between streptokinase (SK), or modified forms of SK, or suitable parts thereof that show an in vitro biological activity that is comparable to that of native streptokinase as measured by plasminogen activation assays, the activity decrease if it occurs in some cases being well compensated by the derivative's extended half-life and/or lower clearance rates.

The invention provides PEGylated cysteine variant(s) of streptokinase that show characteristics of plasminogen activation only after a lag period of more than 5 minutes after exposure of the plasminogen activator to a suitable animal or human plasminogen.

The invention provides prokaryotic or eukaryotic cells, transformed or transfected with expression vectors in which gene to express streptokinase its muteins or covalently modified forms are cloned, and capable of expressing cysteine variants of streptokinase or its muteins or the hybrid plasminogen activators. For efficient expression, the DNA sequences encoding the streptokinase its muteins and covalently modified forms were optimized for codon preferences of bacterial or yeast based expression hosts.

The invention details out a method for the production of PEGylated cysteine variants of streptokinase or its active muteins or the hybrid plasminogen activator molecules in pure and biologically active form for clinical and research applications.

The invention takes into account the PEGylation of those cysteine variants that use template polynucleotide wherein the SK-encoding polynucleotide utilized for expression of SK, is modified, by mutagenesis by known biochemical or chemical DNA synthesis techniques, or their combination such that the plasminogen activator activity is retained.

The invention takes into account cysteine variants of SK or its truncated form/s that are PEGylated but also possess additional fibrin binding domains fused through polypeptide linkages so that the resultant chimeras/fusion polypeptides besides showing plasminogen activation capabilities, also show fibrin binding characteristics. The fusions between the fibrin binding domains and SK can be direct, but may also be through short linker peptide region/s comprising of a stretch of amino acid sequence that is not conformationally rigid but is flexible, such as those predominantly composed of Gly, Ser, Asn, Gln and similar amino acids.

The cysteine variants of SK or its muteins or covalently modified forms are expressed in *E. coli* using standard plasmids under the control of strong promoters, such as tac, trc, T7 RNA polymerase and the like, which also contain other well known features necessary to engender high level expression of the incorporated open reading frame that encodes for the SK or its muteins or covalently modified SK constructs.

The cysteine variants of SK or its muteins or species variants or covalently modified forms are expressed in yeast

expression system using standard plasmids wherein the N-terminal signal peptide is optimized for efficient extracellular secretion of the mature polypeptide. The sequence information for these signal peptides can be obtained from the secretory proteins of yeast expression system. Additionally such information can also be obtained from the other recombinant proteins that are hyper secreted and contain an optimized signal sequence.

The invention provides a method wherein the crude cell lysates obtained, using either chemical, mechanical or enzymatic methods, from cells harboring the single, double or triple cysteine variants of SK or SK chimeric polypeptides are subjected to air or thiol-disulfide reagent catalytic oxidation, or enzyme catalyzed thiol-disulfide oxidative refolding to refold to their biologically active conformations containing the native cysteine pairing (in covalently modified forms of SK) while leaving the additional cysteine(s) free for sulphydryl reactive chemical modifications.

The invention provides a method wherein the crude cell lysates obtained, using either chemical, mechanical or enzymatic methods, from cells harboring the single, double, triple or multiple cysteine variants of covalently modified forms of SK, are subjected to oxidation and refolding using a mixture of reduced and oxidized glutathione, or other such reagents as are useful for such oxidative folding reactions through thiol-disulfide interchange e.g. cysteine and cystine, of a suitable redox potential that allows the covalently modified forms of SK to refold to their biologically active conformations while leaving the additional cysteine(s) free for sulhydryl reactive chemical modifications.

The cysteine variants of SK its muteins or covalently modified forms are expressed in eukaryotic organisms such as yeasts or animal or plant cells using standard genetic methods either as incorporated genetic units in the main genomes, or as autonomous genetic elements well known in the field so as to obtain high level expression of the incorporated open reading frame/s that encode for the SK or its muteins or covalently modified SK constructs.

The invention provides a method wherein a PEGylated cysteine variant of SK or SK chimeric plasminogen activator protein can be used as a thrombolytic therapy or prophylaxis for various vascular thromboses. The activator may be formulated in accordance with routine procedures as pharmaceutical composition/s adapted for administration to human beings, and may include, but are not limited to, stabilizers such as human serum albumin, mannitol etc, solubilizing agents, or anesthetic agents such as lignocaine and the like, as well as other agents or combinations thereof that stabilize and/or facilitate delivery of the variants in vivo.

The invention provides a pharmaceutical composition comprising PEGylated Cysteine variants of SK or hybrid plasminogen activator and stabilizers that include, but are not limited to, human serum albumin, mannitol etc, and solubilizing agents, anesthetic agents etc.

The present invention will be explained in more detail in the following examples that are, however, not intended to limit the scope of the invention. Taking cognizance of the present invention other variants, combinations and improvements will be obvious for the person skilled in the art. Thus, similar work or its careful imitations are likely to generate similar or improved features even in other variants of streptokinase that are not disclosed in this invention and belong to different isolates of human or non-human origin.

## EXAMPLES

### General Methods Used in Examples

In general, the molecular methods and techniques well known in the area of molecular biology and protein science

were utilized. These are readily available from several standard sources such as texts and protocol manuals pertaining to this field of the art, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual* (II. sup. nd edition, Cold Spring Harbor Press, New York, 1989; McPherson, M. J., Quirke, P., and Taylor, G. R., [Ed.] *PCR: A Practical Approach*, IRL Press, Oxford, 1991, *Current Protocols in Protein Science*, published by John Wiley & sons, Inc. For immunological experiments text and protocol manuals from *Immunochimical Protocols*, Hudson L, Hay F C (1989) 3rd ed. Blackwell Scientific was referred.

This however does not limits the detailed explanation in the context of specific experiments describing the present invention, particularly where modifications were introduced to established procedures, are indicated in the Examples whenever relevant.

### Reagents

The cloning of SK gene was done in the T7 RNA polymerase promoter-based expression vector, pET-23d and was transformed in the *Escherichia coli* BL21 (DE3) strain procured from Novagen Inc. (Madison, Wis.). Thermostable DNA polymerase (Pfu), restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were acquired from New England Biolabs (Beverly, Mass.). Oligonucleotide primers were supplied by one of these; Biobasic, Inc., Canada, Integrated DNA technologies, US, or Sigma-Aldrich, US. Purifications of DNA and extraction of PCR amplified products from agarose gels were performed using kits available from Qiagen GmbH (Germany). Automated DNA sequencing using fluorescent dyes was done on Applied Biosystems 3130x1 genetic analyzer 16 capillary DNA sequencer. Glu-plasminogen was either purchased from Roche Diagnostics GmbH (Penzberg, Germany) or purified from human plasma by affinity chromatography (Deutsch and Mertz, 1970). The N-terminal amino acid sequencing was done with Applied Biosystems sequencer, Model 476A and 491 etc. Urokinases, EACA, sodium cyanoborohydride, L-Lysine were purchased from Sigma Chemical Co., St. Louis, USA. Phenyl Agarose 6XL and DEAE Sepharose (Fast Flow) were procured from Pharmacia Biotech, Uppsala, Sweden, while, Ni-NTA beads were from Qiagen. All other reagents were of the highest analytical grade available.

Casein-plasminogen overlay for detection of SK activity: Activity of different SK derivatives were detected by overlay of casein and human Plasminogen in soft agar. The original method of Malke and Ferretti, 1984; was modified where purified SK (0.5 microgram) was directly spotted on marked depressions on LB-Amp agar plates. The plate was then incubated at 37° C. for 10 minutes; thereafter, casein-HPG-agarose was overlaid by gently pouring the mixture of solutions A and B on top of the plate containing the spots. Solution A was prepared by heating 1 g of skim milk in 15 ml of 50 mM Tris, Cl (pH 7.5), after which it was maintained at 37° C. in a water-bath till further use. Solution B was prepared by heating 0.38 g of agarose in 15 ml of 50 mM Tris. Cl (pH 7.5) at 50° C. After tempering the solution to 37° C., 3 µl of TritonX-100 (0.04% v/v) and 200 µg HPG was added. The plate was then incubated at 37° C. and observed for the generation of zones of clearance (halo formation) due to casein hydrolysis, following HPG activation.

For proteolytic stability, each PEGylated derivative and the native SK was incubated with proteolytic enzymes such as trypsin or plasmin. Protease concentrations used in the reaction were varied from 500-10,000 fold of the protein concentration. The reactions were kept under shaking condition at 25° C. for two to four hours. Same amount of trypsinized protein for both test and control were spotted on the LB-Amp

agar plates and the residual activity was measured after Casein-plasminogen overlay of the plates.

SDS-PAGE analysis of proteins: SDS-PAGE is carried out, essentially according to Laemmli, U. K., 1970, with minor modifications, as needed. Briefly, protein samples are prepared by mixing with an equal volume of the 2.times.sample buffer (0.1 M Tris Cl, pH 6.8; 6% SDS; 30% glycerol; 15% beta-mercaptoethanol and 0.01% Bromophenol Blue dye). For non-reducing SDS-PAGE beta-mercaptoethanol was not included in the sample buffer. Prior to loading onto the gel, the samples are heated in a boiling water bath for 5 min. The discontinuous gel system usually has 5% (acrylamide concentration) in the stacking and 10% in the resolving gel. Electrophoresis is carried out using Laemmli buffer at a constant current of 15 mA first, till the samples stack and then 30 mA till the completion. On completion of electrophoresis, gel is immersed in 0.1% Coomassie Blue R250 in methanol:acetic acid:water (4:1:5) with gentle shaking and is then destained in destaining solution (20% methanol and 10% glacial acetic acid) till the background becomes clear.

PEGylated proteins can be additionally stained with iodine through a standard method developed by Kurfurst (Kurfurst M M., 1992) that specifically stains the PEG molecules. For iodine staining of purified PEG variants, briefly after electrophoresis the gel was soaked in a 5% glutaraldehyde (Merck) solution for 15 min at room temperature for fixation. Afterward the gel was stained for PEG as follows. First, the gel was put in 20 ml of perchloric acid (0.1M) for 15 min, and then 5 ml of a 5% barium chloride solution and 2 ml of a 0.1 M iodine solution (Merck, Titrisol 9910) were added. The stained PEGylated protein bands appeared within a few minutes. For dual staining of SDS PAGE the iodine stained gels were further stained by Coomassie Blue 8250 using the protocol of Laemmli as described in [0044].

Kinetic assays (Shi et. al., 1994, Wu et. al., 1987, Wohl et. al., 1980) were used for determining the HPG activation by PEG modified or unmodified SK or its covalent variants especially when kinetic constants were needed to be determined. Varying concentrations of either PEG modified or unmodified SK or its covalently modified forms (10 nM-200 nM) were added to a final volume of 100 microliter in multi-well plate containing 1-2 uM of HPG in assay buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5-1 mM chromogenic substrate and 0.1 M NaCl). The chromogenic substrate used (S-2251, Roche Diagnostics GmbH, Germany) was plasmin specific and gives yellow color product upon cleavage that can be monitored at 405 nm. The protein aliquots were added after addition of all other components into the well and taking the first spectrophotometric absorbance zero. The change in absorbance at 405 nm was then measured as a function of time in a Versa-Max tunable microplate reader from Molecular Devices USA. Appropriate dilutions of *S. equisimilis* streptokinase obtained from WHO, Hertfordshire, U.K. is used as a reference standard for calibration of international units in the unknown preparation.

Assay for determining the steady-state kinetic constants for HPG activator activity of PEG modified or unmodified SK and its covalently modified forms.

To determine the kinetic parameters for HPG activation, fixed amounts of PEG modified or unmodified SK or its covalently modified forms (0.05-0.1 nanomolar) were added to the assay buffer containing various concentrations of HPG (ranging from 0.035 to 2.0 micromolar) in the multi-well plate as described above. The change in absorbance was then measured spectrophotometrically at 405 nm for a period of 10-40 min at 25 C. The kinetic parameters for HPG activation

were then calculated from inverse, Michaelis-Menton, plots by standard methods (Wohl et. al., 1980).

Various PEGylated SK or its muteins and the native SK was radio-iodinated with Iodine-125 (I125) procured from PerkinElmer Singapore Pte Ltd. Using the Iodogen (1,3,4,6-Tetrachloro-3 $\alpha$ -6 $\alpha$ -diphenylglucoluril) method (Fraker & Speck, 1978). According to the method used by Fraker and Speck, the Iodogen is dissolved in chloroform and coated onto the wall of a borosilicate glass tube by evaporating the solvent with spray of nitrogen gas. For iodination, protein solution in Phosphate Buffered Saline (PBS) is added to the Iodogen coated tube and mixed with I125. After approximately 10-30 min, the radio-iodinated protein is separated from free radio-iodine by desalting on a Sephadex G 25 fine matrix containing column (Amersham Biosciences).

#### Genetic Constructs

##### Construction of Streptokinases

The design and construction of the pET vector containing the SK gene (pET-23d-SK) has been described in Nihalani et al., (1998). It involved the cloning of the SK gene from *Streptococcus equisimilis* H46A in pBR 322 (Pratap et al., 1996), followed by subcloning into pET-23d, an expression vector containing a highly efficient ribosome binding site from the phage T7 major capsid protein (Studier and Moffatt, 1986) and further modification of the 5' end of the gene to minimize the propensity for formation of secondary structure. It had an in-frame juxtaposition of an initiation codon for Met at the beginning of the open reading frame encoding SK so as to express the protein as Met-SK. For details reference can be made to Sahni et. al; 2007 (U.S. Pat. No. 7,163,817).

SK muteins were also designed using the refurbished template as in case of SK so to get high intra-cellular expression capability. The circular map of pET-23d-SK has been depicted in FIG. 2. The scheme to make truncated derivatives of SK is otherwise explained in detail by Nihalani et al., 1998. Apart from gene sequencing, the authenticity of SK and its truncated derivatives were also established by gas phase N-terminal amino acid sequencing of proteins on an Applied Biosystems-Perkin Elmer protein sequencer model 476A or 491 clc.

Construction of covalently modified forms of SK by making a hybrid DNA polynucleotide between SK-encoding DNA and fibrin binding domains of human fibronectin and its cloning and expression in *E. coli* are explained in detail in U.S. Pat. No. 7,163,817. Briefly the fibrin binding domains are fused to streptokinase at N-terminus or at C-terminus or both at N and C-terminus to generate various covalently modified forms of streptokinase.

#### Example 1

##### Selection of Residues or the Regions of Protein for Generating Cysteine Mutants of Streptokinase

Residue selection for substitution or deletion is crucial to maintain the functionality of modified polypeptides. Therefore, cysteine mutagenesis plan requires both structural information present in crystal structure and the functional insights obtained through solution studies. Extensive structure and function studies over the years has gathered tomes of information about the role of different regions of streptokinase in plasminogen activation. To decide upon the residues or the region where the naturally present amino-acid can be preferably substituted with the cysteine, we utilized information present in three dimensional structure of SK or its isolated domain along with their functional relevance. The selection of residues for cysteine mutagenesis was partly based on the

determination of the surface accessibility of the residues. Site of cysteine insertion was also limited to flexible regions of the streptokinase. To determine the surface exposure DSSP program was used. The DSSP code is frequently used to describe the protein secondary structures with a single letter code. DSSP is an acronym for "Dictionary of Protein Secondary Structure", The DSSP (Kabsch and Sander, 1983) program defines secondary structure, geometrical features and solvent exposure of proteins, given atomic coordinates in Protein Data Bank format. DSSP states each residue's exposure in terms of square .ANG.ngstroms. Run of the DSSP program on a given PDB file produce abbreviated DSSP format output. One can get the value of surface accessibility under the heading Acc in the DSSP format output. To determine the surface exposure, solved crystal structure of streptokinase (Wang et. al. 1998, PDB ID 1BML) in complex of microplasmin was used. For the regions that were missing in this structure (175-181 and 252-262) crystal structure of the isolated beta domain (Wang et. al., 1999, PDB ID 1c4p) was used for determination of surface exposure. Some of the loops missing in the crystal structure were grafted in the experimentally obtained structure and there most preferred conformation was determined through molecular modeling tools. Residues not necessarily detected in the structure but are defined as highly accessible as they reside in the flexible region were also chosen for cysteine mutagenesis. Table 1 shows the surface accessibility values for different cysteine variants of SK (SEQ ID NO: 1) that were substituted with cysteine. The list however, does not limit the scope of cysteine substitution for the other naturally present amino-acids of SK. The accessibility values calculated by the program DSSP were directly taken as the measure of surface exposure. The DSSP program listed many surface exposed residues. But a careful selection was done while deciding the residues for cysteine substitution. This exercise included mutations evenly spread all along the three different domains i.e. alpha, beta and gamma of streptokinase. Mutations were also selected that fall in the secondary structural regions. Selection also included cysteine replacement of few residues that show exceptionally low surface accessibility just to ensure the fact that in principal each and every residue of the streptokinase can be replaced with cysteine and successfully modified with the thiol reactive reagents.

Despite of nucleotide and polypeptide sequence diversity, there exists a strong structural similarity among different bacterial plasminogen activators. The one-domain staphylokinase bears structural homology with the alpha domain of streptokinase. Also the two-domain bovine plasminogen activator obtained from *Streptococcus uberis* shows structural similarity with alpha and beta domains of streptokinase. Evolutionary conservation of protein three dimensional structure among different bacterial plasminogen activators makes it feasible to plan cysteine modifications of other streptokinase variants that are isolated from different bacterial species.

#### Example 2

##### Genetic Construction of Cysteine Variants of Streptokinases

All the genetic constructs to express streptokinases were generally constructed by using conventional approaches known in the art. The methods of DNA manipulation to incorporate mutations are described, for example, in TCR Protocols: A Guide to Methods and Applications', edited by Innis, M. A. et al. 1990., Academic Press Inc., San Diego, Calif. and TCR Protocols: Current Methods and Applications' edited by B. A. White, 1993., Humana Press, Inc., Totowa, N.J., USA.

Bacterial and Yeast expression cassettes were made by inserting the DNA molecule encoding the desired streptokinases into a suitable vector (or inserting the parent template DNA into the vector and mutagenizing the sequence as desired therein), then transforming the host cells with the expression cassette using conventional methods known in the art. Specific mutations were introduced into the desired constructs using a variety of procedures such as PCR mutagenesis techniques (Innis et. al., 1990), mutagenesis kits such as those sold by Stratagene ("Quick-Change Mutagenesis" kit, San Diego, Calif.) or Promega (Gene Editor Kit, Madison Wis.). In general, oligonucleotides were designed to incorporate nucleotide changes to the coding sequence of Streptokinases that result in substitution, deletion or addition of desired residue for the naturally present residue. Mutagenic primers were also designed to add the cysteine at the beginning of the mature protein, i.e. proximal to the N-terminal amino-acid or following the last amino acid in the mature protein, i.e. after the C-terminal amino-acid of the Streptokinase and its truncated constructs. Similar strategy was used for insertion of cysteine residues between any two selected amino-acids of the Streptokinase or any of its form represented by SEQ IDs present in Table 2. Using the standard methods, corresponding cysteine containing mutants were generated on various forms of SK. The transformed clones for different mutants were then screened and confirmed by automated DNA sequencing using fluorescent dyes on an Applied Biosystems 3130x1 genetic analyzer 16 capillary DNA sequencer.

Table two lists different polypeptide constructs expressing one of the followings: streptokinase; its muteins; species variants; or covalently modified forms. The native full length polypeptide sequence of streptokinase has been assigned SEQ ID NO: 1. The truncated form of SK where C-terminal 31 residues are deleted is depicted by SEQ ID NO: 2. The truncated form of SK where N-terminal 15 residues are deleted is represented by SEQ ID NO: 3. Polypeptide that contains deletion of both N-terminal 15 residues and C-terminal 31 residues of SK is given SEQ ID NO: 4. Functional fragment of SK where the N-terminal 49 residues are deleted has been assigned SEQ ID NO: 5. Streptokinase construct in which both N-terminal 59 and C-terminal 31 residues are deleted has been given SEQ ID NO: 6. Full length polypeptide of SK (residues 1-414) that contains alanine substitution for Asparagine 90 in the alpha domain is represented by SEQ ID NO: 7. Beta domain mutant polypeptide of full length SK that has substitution of Tyrosine in place of Alanine has been given SEQ ID NO: 8. Similarly SEQ ID NO: 9 represents beta domain mutant of SK where Aspartate residue at 238th position is substituted with Alanine. SEQ ID NO: 10 is assigned to beta domain mutant of streptokinase where Glutamate at 240th position is substituted with Alanine. SEQ ID NO: 11 and SEQ ID NO: 12 represent Arginine to Alanine, and Lysine to Alanine mutation at 244th and 246th residues respectively, in full length SK. The 250 loop mutant of beta domain of SK where Leucine residue at 260th position is substituted with Alanine is given SEQ ID NO: 13. The gamma domain mutant of SK where Aspartate residue at 359th position is substituted with Arginine is represented by SEQ ID NO: 14. The double mutant of SK where both Histidine 92 and Serine 93 are substituted with Alanine is represented by SEQ ID NO: 15. Another double mutant of SK, where two consecutive Lysine residues at 278th and 279th position were substituted by Alanine is represented by SEQ ID NO: 16. The mutants of SK where Asparagine at 90th position in alpha domain, Aspartate at 227th position in beta domain or Aspartate at 359th position of gamma domain have been deleted are given SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19

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respectively. Matured and active forms of SK are available from a number of species and subspecies *variants* of the genus *Streptococcus*. To validate the feasibility of cysteine mutagenesis and subsequent PEGylation across the different forms of SK, we also selected variants of SK derived from *Streptococcus* species, namely *pyogenes* and *dysgalactiae*. The SK species variant derived from *Streptococcus pyogenes* is given SEQ ID NO: 20 and the one obtained from *Streptococcus dysgalactiae* is given SEQ ID NO: 21. The covalently modified form of SK where fibrin binding domains are present at the N-terminus of SK is given SEQ ID NO: 22. The C-terminus fibrin binding domain fusion product of SK has been assigned SEQ ID NO 23. Hybrid polypeptide that contain fibrin binding domain both at N and C-terminus of SK is given SEQ ID NO: 24. Genetic constructions of fibrin domain fused forms of SK are detailed out in U.S. Pat. No. 7,163,817. Different polypeptides that include native full length SK, its muteins, species variants and the covalently modified forms were further used for generation of cysteine variants.

Cysteine variants generated on different forms of SK mentioned in TABLE 2 have been assigned unique SEQ IDs. Table 3 to 28 lists individual variants along with their unique SEQ IDs.

Table 3: variants those were designed on native full length SK (SEQ ID NO: 1)

Table 4: variants those were designed on truncated SK 1-383 (SEQ ID NO: 2)

Table 5: variants those were designed on truncated SK 16-414 (SEQ ID NO: 3)

Table 6: variants those were designed on truncated SK 16-383 (SEQ ID NO: 4)

Table 7: variants those were designed on truncated SK 50-414 (SEQ ID NO: 5)

Table 8: variants those were designed on truncated SK 60-383 (SEQ ID NO: 6)

Table 9: variants those were designed on mutant SK polypeptide (SEQ ID NO: 7)

Table 10: variants those were designed on mutant SK polypeptide (SEQ ID NO: 8)

Table 11: variants those were designed on mutant SK polypeptide (SEQ ID NO: 9)

Table 12: variants those were designed on mutant SK polypeptide (SEQ ID NO: 10)

Table 13: variants those were designed on mutant SK polypeptide (SEQ ID NO: 11)

Table 14: variants those were designed on mutant SK polypeptide (SEQ ID NO: 12)

Table 15: variants those were designed on mutant SK polypeptide (SEQ ID NO: 13)

Table 16: variants those were designed on mutant SK polypeptide (SEQ ID NO: 14)

Table 17: variants those were designed on mutant SK polypeptide (SEQ ID NO: 15)

Table 18: variants those were designed on mutant SK polypeptide (SEQ ID NO: 16)

Table 19: variants those were designed on mutant SK polypeptide (SEQ ID NO: 17)

Table 20: variants those were designed on mutant SK polypeptide (SEQ ID NO: 18)

Table 21: variants those were designed on mutant SK polypeptide (SEQ ID NO: 19)

Table 22: Cysteine variants of *Streptococcus pyogenes* MGAS10270 (SEQ ID NO: 20)

Table 23: Cysteine variants of *Streptococcus dysgalactiae* subsp. *equisimilis* (SEQ ID NO: 21)

Table 24: Cysteine variants of SK with N-terminal fused fibrin binding domain (SEQ ID NO: 22)

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Table 25: Cysteine variants of SK with C-terminal fused fibrin binding domain (SEQ ID NO: 23)

Table 26: Cysteine variants of SK with both N and C-terminal fused fibrin binding domains (SEQ ID NO: 24)

Table 27: Cysteine insertion mutants of SK

Table 28: variants of SK where cysteine is placed at the N or C-termini with or without a peptide extension.

These examples demonstrate that one can generate cysteine variants on virtually all forms of SK such as native full length, truncated, N or C terminally extended or in fusion with other polypeptide sequence. We also generated cysteine variants of substitution, insertion or deletion mutants of SK. This validates the applicability of this invention to any form of SK for cysteine mutagenesis and subsequent modification with thiol reactive agents.

The constructs obtained from Example 2 were utilized in all further experiments conducted to arrive at the present invention. However, it should be understood that the list of cysteine variants of streptokinases are merely exemplary and not exclusive. The design and synthesis of alternative and additional cysteine variants of streptokinases in accordance with this invention are well within the present skill in the art. Synthesis of such variants may be conveniently effected using conventional techniques and methods.

## Example 3

## Over-Expression and Purification of Biologically Active Streptokinases

The native streptokinase protein (nSK), its mutants and their subsequent Cysteinylyl mutants to be purified were each grown from single colony, streaked on LB-Amp plate from their BL21 (DE3) glycerol stocks. The primary cultures were developed by inoculating pET-23d-SK or SK variants into 10 ml of LB medium containing 100 microgram/mL ampicillin (LB-Amp medium) and incubated for 8-16 hours at 30-37 C, under shaking conditions (180-280 rpm). This pre-inoculum was used to seed 500 ml of LB-Amp medium at 2-10% v/v and allowed to grow at 30-37 C., at 180-280 rpm to an O.D600 nm (optical density measured at 600 nano-meter) of 0.5-1.0. At this stage, it was induced with IPTG (final concentration of 0.5-1.0 millimol) (Chaudhary et al., 1999; Dhar et al., 2002) and further grown at 40 C, for 6-12 hours under shaking condition. Cells were then harvested by centrifugation at 6000-7000 g for 10 min. The pellet was then washed twice with ice-cold buffer (final concentrations-100-150 mM NaCl, 10-50 mM Tris-Cl, pH 8.0, and 1-5 mM EDTA) and subjected to sonication (Heat System, New York) at 4 C, under conditions of 30 sec sonic-pulses interspersed with equal periods of rest. The cell lysate was then centrifuged at high rpm (10000-14000 g) for 15 min. The SDS-PAGE analysis show that more than 90% desired protein had gone to the Inclusion Bodies (IBs). The IBs were then solubilised in 8 Molar urea at room temperature for 45 min under constant gentle shaking condition. The protein in supernatant was folded after 10-fold dilution (Sundram et al., 2003) in the loading buffer (0.4 M NaCl in 20 mM PB). The sample was then chromatographed on Phenyl Agarose 6XL beads and eluted in water. The protein so obtained was then subjected to further purification by anion-exchange chromatography on a DEAE-Sepharose column (GE-Amersham Biosciences). Protein fractions after HIC were pooled and Tris. Cl pH 7.5 was added to a final concentration of 20 mM Tris. Cl, after which it was loaded onto a column packed with DEAE-Sepharose (Fast Flow) pre-equilibrated with 20 mM Tris. Cl (pH 7.5). After washes with buffer containing 20 mM Tris. Cl (pH 7.5), the bound



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protein was eluted using a linear gradient of salt (0-0.5 M NaCl) in 20-25 mM Tris. Cl. SK proteins eluted were generally more than 95% pure, as analyzed by SDS-PAGE. The amount of protein in each fraction was measured using Bradford's method of protein estimation (Bradford., 1976) and confirmed by Absorption at 280 nm. All chromatographic steps were conducted at 4.degree. C. The fractions containing protein were analyzed on SDS-PAGE along with standard SK and Molecular mass markers. Desired fractions were pooled conservatively to obtain the homogenous preparation of SK or SK mutants.

Over expression and purification of various covalently modified constructs formed by SK and Fibrin Binding Domains (FBDs) in *E. coli* and their in vitro refolding are described in U.S. Pat. No. 7,163,817 wherein the expressed proteins were subjected to in vitro refolding and purified by column chromatography. Briefly, the solubilized inclusion bodies were diluted to a final protein concentration of 1 mg/ml using distilled water; together with the addition of the following additional components (final concentrations in the diluted mix are given): Tris-Cl, pH 8.0, 50 mM; NaCl 100-150 mM; EDTA 1-5 mM; mixture of reduced and oxidized glutathione 1.25 mM:0.5 mM. The refolded population was separated and purified on a column packed with fibrin-sepharose beads. For detailed description of the refolding, purification and characterization of the refolded protein please refer to Sahni et. al; 2007 (U.S. Pat. No. 7,163,817).

## Example 4

## Covalent Conjugation of Cysteine Variants of Streptokinases with Polyethylene Glycol

The thiol groups of the cysteine variants of streptokinases were selectively PEGylated using maleimide-activated linear methoxy PEG of different sizes such as 5 KDa, 20 KDa and 40 KDa (JenKem Technology, USA). For the PEGylation reaction the polypeptide to be PEGylated was kept in 50-100 mM Tris-Cl buffer pH 8.0 containing 100-150 mM of NaCl. To this, 5 molar excess of PEG reagent was added. The molar excess was calculated while taking into consideration the number of free thiols to be reacted with PEG reagent and not merely the protein's molarity. The reaction mix was allowed to stir at room temperature for 1.5-4 hours, and then the reaction was stopped by adding 1 mM of DTT. PEGylated protein from the free PEG and the unreacted SK was purified by anion exchange chromatography on a DEAE sepharose column (GE-Amersham Biosciences). The reaction mixture was diluted 10-15 fold with 25 mM Sodium Phosphate buffer of pH 7.4 after which it was loaded onto a column packed with DEAE-Sephacrose (Fast Flow) pre-equilibrated with the same buffer. After washes with buffer containing 25 mM Sodium Phosphate, the bound protein was eluted using a linear gradient of salt (0-0.5 M NaCl) in 25 mM Sodium Phosphate. Alternatively, if some of the PEGylated derivatives failed to separate from unreacted PEG cleanly by ion-exchange, these reactions were subjected to size-exclusion chromatography on Sephadex 75 (Amersham Biosciences) using a buffer of neutral pH and final NaCl concentration of 100-150 mM. Also, in some cases, where purified cysteinylated protein samples that showed disulfide bonded dimers were first reduced by addition of 10 mM DTT. The DTT treated samples were desalted on a column packed with Sephadex G-25 (fine) beads, and immediately used for PEG conjugation. The homodimeric forms of SK due to intermolecular disulfide linkage can also be separated using size-exclusion chroma-

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tography on Sephadex 75 and may be useful over monomeric streptokinase for therapeutic uses due to its large size and slow clearance.

PEG cross-linking in all cases was confirmed by SDS PAGE. Gel electrophoresis showed >95% of the PEGylated protein in the fractions that were obtained after removal of the unreacted protein and the free PEG reagent. FIG. 1 shows few mono and bi-PEGylated variants of SK. Panel A shows one of the representative (D95C, SEQ ID NO: 30) PEGylated cysteine variant of the alpha domain of streptokinase where naturally present aspartate residue has been replaced with cysteine and conjugated with methoxy PEG maleimide of molecular mass 20 KDa. Panel B depicts one of the representative (S258C, SEQ ID NO: 49) PEGylated cysteine variant of the beta domain of streptokinase where naturally present serine residue in the 250 loop of beta domain has been replaced with cysteine and conjugated with methoxy PEG maleimide of molecular mass 20 KDa. Panel C shows one of the representative (C-Cys, SEQ ID NO: 490) PEGylated cysteine variant of the gamma domain of streptokinase where one cysteine has been placed at the C-terminus of the SK and conjugated with methoxy PEG maleimide of molecular mass 20 KDa. Panel D shows one of the bi-PEGylated cysteine variant of SK (SEQ ID NO: 493) where one cysteine is placed each at the N-terminus and C-terminus of the molecule and conjugated with methoxy PEG maleimide of molecular mass 20 KDa. Conjugation of PEG at the two extremes of SK was carried out with the premise that two bulky PEG groups will enclose the protein in a flexible cocoon and extend its in vivo survival for prolong period. At the same time extensive masking of the immuno-dominant regions may attract a negligible immuno-reactivity of the injected molecule in a subject. Fractions containing PEGylated protein were pooled and formulated for activity assays, and in some instances were further characterized by MALDI-TOF mass spectrometry. The mass spectrometry also confirmed the expected molecular mass of PEG adduct of streptokinase and its covalently modified forms.

## Example 5

## Casein-Plasminogen Overlay for Rapid Detection of Plasminogen Activation Ability

Activity of different cysteine variants of SK and SK chimeras were detected by overlay of casein and HPG in soft agar. The original method of Malke and Ferretti, 1984 was modified where purified SK (0.1-0.5 microgram) was directly spotted on marked depressions on LB-Amp agar plates. The plate was then incubated at 37.degree. C. for 10 minutes; thereafter, casein-HPG-agarose was overlaid by gently pouring the mixture of solutions A and B on top of the plate containing the spots. Solution A was prepared by heating 1 g of skim milk in 15 ml of 50 mM Tris, Cl (pH 7.5), after which it was maintained at 37.degree. C. in a water-bath till further use. Solution B was prepared by heating 0.38 g of agarose in 15 ml of 50 mM Tris, Cl (pH 7.5) at 50.degree. C. After tempering the solution to 37.degree. C., 3.mu.l of TritonX-100 (0.04% v/v) and 100-200 .mu.g HPG was added and gently mixed without frothing. The plate was then incubated at 37.degree. C. for 1-4 hrs and observed for the generation of zones of clearance (halo formation) due to casein hydrolysis, following HPG activation. The area of the lysis zone (halo) surrounding the well into the agarose medium was taken into consideration for comparing the plasminogen activation ability for the streptokinase and its covalently modified variants.



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All cysteine variants of streptokinases retained substantial plasminogen activation ability as examined by Casein-HPG overlay assay. Cysteine variants of streptokinases were further taken for PEG conjugation with thiol reactive PEG of different molecular weights ranging from 5000 Dalton to 40,000 Dalton and their activity was determined using Casein-HPG overlay assay with activity of PEG conjugated with the justification that a loss in plasminogen activity ability All PEG modified variants of streptokinases showed substantial plasminogen activation ability under caseinolytic assay and are therapeutically useful. However, a desired combination of suitable half-life that is clinically required along with reduced immune reactivity, make some derivatives more useful over others.

Comparatively reduced activity in some cases may well be compensated by increased proteolytic stability and in vivo half-life of the PEG-protein adducts.

Alternatively, the plasminogen activation ability were also measured as explained previously. Table 29 shows the HPG activator activity and the kinetic constants for a few representative PEGylated SK variants. Table 30 summarizes the range of activity for the different PEGylated variants of covalently modified streptokinases that contain fibrin domain fusion.

The activity measurement for truncated form of SK (50-414 and 60-383) and their PEGylated variants required supplementation of synthetic peptides 1-49 for optimal amidolytic and plasminogen activation capabilities. It has been documented in the literature that truncated variants of SK that are devoid of N-terminal peptide corresponding to 1-59 region are poor plasminogen activators and show increased activity upon supplementation of either SK 1-59 peptide or fibrin in the reaction mixture for optimal amidolytic and plasminogen activation capabilities (Nihalani et. al., 1998 and Sazonova et. al., 2004). PEGylated derivatives of SK that does not contain 1-49 or shorter N-terminal peptides show fibrin dependence for plasminogen activation hence; their actions are restricted to clot mainly making them clot-specific.

## Example 6

## Proteolytic Stability of PEGylated Cysteine Variants of Streptokinases

For proteolytic stability, 50 microgram of each PEGylated derivative and the native SK, as control, was incubated with 50 microliters of 50 mM Tris-Cl and 100 mM NaCl. To this, trypsin was added to give a final ratio of PEGylated Protein: Trypsin of 1000:1 (w/w). The reaction was kept under shaking condition at 25° C. for two to four hours. Equal aliquots of trypsinized protein was spotted on the LB-Amp agar plates and the residual activity was measured in a similar way as explained in EXAMPLE 5 for detection of SK activity. Equal aliquots were also spotted from the control reactions where only trypsin was added in the reaction or only SK or SK hybrids were added in the reaction. In this case also the zone of lysis on agarose overlay was measured for each of the trypsinized PEGylated streptokinase or its covalently modified variants. The area of zone of lysis becomes a direct measure of the residual plasminogen activation ability for the protected streptokinase or its variants. This assay showed significant protection for the different SK variants under this study when they were conjugated with single, double or triple PEG moieties and incubated with trypsin or plasmin. We found a multiplied increase in proteolytic stability with attachment of more than one PEG group to the SK variants.

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Alternatively, the residual activity measurements were also confirmed for trypsinized PEGylated streptokinase or its covalently modified variants by testing their Plasminogen activation capability using the one-stage assay described earlier using chromogenic substrate spectrophotometrically [0046]. The trypsinized SK and PEGylated SK variants at varying concentrations (1-10 nM) were added to a final volume of 100 microliters in a multi-well plate containing 1-2 uM of HPG in assay buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5-1 mM chromogenic substrate and 0.1 M NaCl). The protein aliquots were added after addition of all other components into the well. The change in absorbance at 405 nm was then measured as a function of time in a Versa-Max model tunable microplate reader from Molecular Devices Inc., USA. Results obtained through this method were in consonance with those obtained through caseinolytic assay. It was found that significant functional activity retained in all the PEGylated forms of streptokinase and its covalently modified form when incubated with trypsin. The unpegylated streptokinases when subjected to similar conditions show barely detectable activity and are prone to trypsin digestion.

Samples subjected to trypsinization were also examined on reducing SDS-PAGE to physically observe the proteolytic stability, and the generation of truncated fragments as a result of the proteolysis. This gave a qualitative assessment of protected protein subjected to trypsinization. For this, aliquots were taken at different time-intervals from the reaction mixture and inhibited by the addition of 20 molar excess of Soybean Trypsin Inhibitor (GE-Amersham Biosciences) to stop any further tryptic activity. Samples collected at different time points (5-180 min) were electrophoresed on 10% SDS-PAGE and analyzed for the protected intact protein. Results obtained from this exercise also substantiated our functional examination of the trypsinized proteins. More residual plasminogen activation ability under assay conditions also reflected in more protection of the protein when examined for physical intactness on the SDS-PAGE.

## Example 7

## N and C-Terminally Extended SK Variants and their PEG Modified Forms

In order to establish that an arbitrary extension of few amino-acids both at N or C-terminus of streptokinase or its variants will invariably produce the same result as that obtained with their unextended counterpart, the SK or its cysteine variants were modified either at N-terminus or C-terminus with small amino-acid extensions.

N-terminal extended forms were made using two different strategies giving a polypeptide of two different lengths viz. one with 6 amino-acid extension and another other with a 20 amino-acid extension. Using the overlap extension strategy 18 nucleotide extension coding for 6 histidine residues were placed before the N-terminal amino-acid of the mature streptokinase. The product of this modification was a N-terminally extended protein with additional six amino-acids. To incorporate the 20 amino-acid extensions the cassettes encoding the SK or its variants were transformed from pET 23d to pET 15b (Cat. No. 69661-3, Novagen, Inc. US). Placing the cassette into the pET 15 b gave an N-terminal extension of 20 amino-acids that include a stretch of six histidine residues and a thrombin cleavage site. The cleavage of N-terminal extension from the polypeptide can be effected with thrombin. This removes the stretch of Histidine tag and yields a processed polypeptide with amino-acid sequence of SK only. SEQ ID

NO 496 shows the amino-acid sequence of SK that was obtained due to the cloning in pET 15b.

To generate the C-terminal extended product, a stretch of six histidine residues was added just after the last amino-acid of SK or its variants using the overlap extension strategy. This resulted in placement of additional six residues at the C-terminus. The proteins were purified either using the metal affinity chromatography or the purification methods explained in Example 2 to obtain a homogeneously purified product. Subsequently these purified N or C-terminally extended products of SK or its variants were modified with PEG using the chemistry explained in Example 4. Biochemical characterization for functional activity and the proteolytic stability yielded similar result as those obtained with their unextended counterparts. This gave a strong evidence for the conclusion that other N or C-terminus extended products of SK or its variants would yield similar results. A skilled artisan can think of innumerable possibilities of extending both N and C-terminus of SK or its variants to yield functional forms of streptokinase that can be used for cysteine substitution, insertion or addition and their subsequent thiol modifications with PEG or other sulfhydryl reactive agents.

#### Example 8

##### Assigning New Functional Attributes to the SK Variants where PEG Groups are Attached at the Two Termini i.e. N and C-Terminus of SK or its Truncated Variants

It was observed that addition of PEG groups simultaneously at both N and C termini of streptokinase and any of its truncated functional variants under study makes its activity dependent on presence of plasmin. This new functional attribute was assigned when we observed that bi-PEGylated SK variant shows a lag (see FIG. 3) of several minutes in the plasminogen activation profile. To our surprise, when the bi-PEGylated SK variant was examined for plasminogen activation after it is complexed with plasmin, it showed normal (i.e. native SK like) kinetics of plasminogen activation (see FIG. 4). The inability to be self-activated immediately (as is the case with native, unmodified SK which activates PG virtually upon contact) is due to a plasmin-dependent mode of its action. In contrast, native SK does not require any plasmin to be activated, but is activated virtually as soon as it complexes with PG. In experiments where samples were withdrawn from reactions at different time points in the progress curves of plasminogen activation i.e. during the early phase (in the lag period), rapid activation phase etc, and analyzed with SDS-PAGE to follow the type of products, it was observed that the activation closely followed the generation of truncated fragments in which the PEG containing peptide segments at the ends of the polypeptide were cleaved off by the action of plasmin. Similar results were obtained by Mass spectroscopic analyses. These results clearly established a correlation between plasminogen activation by the PEG-modified SK upon the elimination of the PEG groups, indicating a plasmin-mediated mechanism of activation wherein the bulky PEG groups, once removed, allowed the SK fragments to interact with plasminogen just as nSK and activate it rapidly. Clearly, therefore, the presence of the PEG groups at the termini—apart from conferring a stabilizing influence against proteolysis, immune reactivity etc expected irrespective of position—also resulted in the unexpected creation of a plasmin dependent “switch”, which has powerful beneficial effects in thrombolytic therapy.

The functional attribute of plasmin dependency i.e. in-built “plasmin switch” was found among other bi-PEGylated cysteine variants of truncated SK, where additional cysteines are placed at the two extremities of the polypeptide i.e. at the N- and C-termini, which exhibits an unexpected property in respect to its human plasminogen activation characteristics in that it has a markedly slower initial rate of activation of plasminogen (PG) compared to unmodified SK, but becomes fully capable of activating plasminogen in a manner similar to that of unmodified SK after an initial lag of several minutes’ duration when assayed for PG activation in vitro. Table 4 shows the steady-state kinetics parameters for HPG activation by SK and the two different bi-pegylated SK variants. NC 1-414 denotes SK variant where cysteine has been added both prior to naturally present N-terminal amino-acid and after the C-terminal amino-acid to generate a double cysteine mutant of SK. NC 1-383 denotes the truncated variant of SK where one cysteine each has been added prior to the naturally occurring N-terminal amino-acid, and after the three consecutive glycine residues that are placed next to the 383rd amino-acid. The data shows that both the bi-pegylated variants show a pronounced initial lag before they become fully functional. The kinetic parameters, when calculated from the linear phases of the reaction progress curves after the abolishment of the lag phase, showed that once fully activated after completion of the initial lag, both bi-pegylated variant became significantly active in terms of their PG activation abilities when compared to SK. Similar results were obtained with two bi-pegylated SK variant where the PEG is attached at the termini, showing that this new functional attribute is positional in effect and not merely dependent on the presence of dual PEG modification within the same molecule. Thus, plasmin dependency is imparted into the molecule whenever either or both of the two termini at the N- and C-termini, in any functional fragment of SK are PEGylated.

In addition, it will be evident to a person skilled in the art that such a functional attribute can also be imparted to the molecule by any modification in and around the two termini (such as a suitable lysine side-chain) would also lead to a plasmin dependent lag in plasminogen activation characteristics owing to proteolytic processing of the streptokinase wherein such “blocking” groups which may be PEG moieties, other protein domains, intact proteins such as albumin etc are removed by proteolysis, allowing the remainder of the polypeptide to become functionally active vis-a-vis plasminogen activation. Similar effects are therefore to be expected when one attaches the PEG groups at the termini using whatsoever chemistry is available. One such chemistry utilizes the differential pKa value of the alpha amino group at the N-terminus to specifically conjugate amine reactive PEG groups at the alpha amine. Similar attributes with different truncated combinations of termini indicate that as long as the two PEG groups are placed at any location in or around the two termini, the plasmin dependency can be generated in the molecule.

Thus, after injection into the body, such a SK variant will make its voyage through the vascular system while still in an inactive, or partially active, state. However, it will preferentially become activated in the immediate vicinity of the clot the moment it contacts the clot, which is known to be plasmin-rich whereas the general circulation is not (free plasmin being rapidly inactivated in the “open” circulation due to the presence of plasmin-specific Serpins (serine protease inhibitors) such as alpha-2-antiplasmin and alpha-2-macroglobulin), thereby obviating or significantly minimizing the systemic PG activation coincident with natural SK administration which immediately activates PG upon administration and consequent side-effects such as hemorrhage and large scale

destruction of various protein components of the vascular system. This property i.e. plasmin-dependant activation, along with the extended elimination half-life, and low immunogenic and antigenic reactivity would result in not only an overall diminished generation of free plasmin in the general circulation but also the ability for the thrombolytic to be administered repeatedly for various circulatory maladies in a relatively lower dose while avoiding unwanted immune reactions. The net result shall be a continued and more efficient fibrinolysis at the target sustained by considerably lowered therapeutically effective dosages of the thrombolytic agent with minimized side-effects.

A further improvement over this attribute is to impart the fibrin dependency in the bi-PEGylated molecule by placing the PEG groups at the two extremes of SK 50-414 or SK 60-383 variants. This outcome is possible because deletion of a "catalytic switch" (SK residues 1-59) alters the conformation of the SK alpha domain and converts such truncated fragments into a fibrin-dependent plasminogen activator as reported by Reed et. al., 1999 and Sazonova et. al., 2004. The expected outcome of designing such bi-PEGylated variants is both a plasmin dependency and an improved fibrin dependency/affinity. These two attributes i.e. plasmin dependency and the fibrin affinity/selectivity makes the molecule a PG activator that is highly directed towards fibrin clots. Such molecules can effectively obviate the problem of systemic PG activation and allow a stringent PG activation to occur only in the near vicinity of the fibrin clot.

#### Example 9

##### Pharmacokinetic Analysis of PEGylated Cysteine Variants of Streptokinases

All proteins used for injection in animal were treated thoroughly to remove endo-toxin by passage through a column consisting of Polymyxin B Agarose (BioRad Inc., Palo Alto, Calif., USA) gel. Various monoPEGylated SK derivatives representing cysteine incorporation in each domain, and the native SK (as control) were radio-iodinated with 125I using the Todogen (Fraker & Speck, 1978) method, and separated from free radio-iodine by passing through Sephadex G-25 desalting column. CD1 mice (23-25 g) were anaesthetized with 3% iso-fluorane and mild vasodilation was induced by exposing the tail to a 100 watt fluorescence lamp. Mice were then injected with around 7 microgram of radio-iodinated protein in sterile saline, via the tail vein, and whole blood samples of approximately 50 microL were collected over time using tail transaction or from retro-orbital sinus and kept in heparinized eppendorf tubes. Samples were processed to yield plasma and were evaluated for 125I activity in a Perkin-Elmer-scintillation counter. After determination of plasma 125I activity, an equal volume of 20% TCA was added to each plasma aliquot, to determine the amount of 125I activity that remained associated with intact protein. The samples were briefly vortex-mixed and were placed on ice for 15 min. The aliquots were centrifuged at approximately 3000.times.g for 10 min, and the supernatant, containing free label or label associated with fragmented protein, was aspirated from each sample. The resultant TCA-precipitated pellet was analyzed for 125I activity. In general, duplicate samples were processed and the values were averaged.

Residual acid-precipitable radioactivity in different plasma samples following injection for PEGylated cysteine variants of streptokinases were used for of in vivo half life determination. Results of half-life study show variable degrees of in vivo retention for the different PEG variants

when they were conjugated with single or double or triple PEG moieties. The results show that with the addition of more than one PEG group the resultant half-lives are also amplified. The half-life for few selected PEGylated SK variants are summarized in Table 32, which shows that on an average there is 5 to 120 fold increase in the elimination half-life for the different mono, bi and tri-PEGylated cysteine variants of streptokinases. Plasma retention times of PEGylated cysteine variants of SK are found to be dependent on the position of PEG attachment. Alpha 88-97 loop PEG variants show maximum increase (.about.15-20 fold) in in vivo retention time while beta domain PEG variants of SK show intermediate increase (.about.10-12 fold) in the in vivo retention. Gamma domain PEG variants show nearly 4-5 fold increase in the in vivo half life. In general, all PEGylated streptokinases showed multifold increase in the half-life when compared to native SK (12-15 minutes). This signifies a considerable increase in the in vivo half-life of the PEGylated cysteine variant, and demonstrates the extended time-action of the PEGylated SK. Similar results were obtained with the other cysteinyl SK variants after PEGylation. It is well known that an extended time-action of the PEGylated variant is a result of the PEG moiety and is not dependent on the location of the PEG polymer on the SK. Thus, attaching the PEG moiety via a cysteine residue will result in a PEGylated SK or SK chimeric variant with extended time-action characteristics allowing for fewer administration of the PEGylated compound while maintaining a high blood level of the compound over a prolonged period of time.

Additivity in the elimination half-life when two PEG groups are attached shows that one can manipulate at will, or tailor-make, the elimination half-life by conjugation of PEG groups in required number, position and also by varying the PEG polymer size.

#### Example 10

##### Immune Reactivity of PEGylated Cysteine Variants of Streptokinases

Reactivity of nSK and PEGylated cysteine variants of SK and its covalently modified forms against SK (polyclonal) anti-sera raised in rabbit was examined by an ELISA-based method. The procedure for ELISA was as follows.

1. SK and PEGylated SK variants were first diluted in 0.2M Bicarbonate buffer, pH 9.2 to make 100 microliter of solution containing 0.75 microgram to 1.5 microgram of protein and this was added to each well of the microtiter plate (Nunc 96-Well Microplates, Cole-Parmer USA)

2. The antigen coated plate was covered with Paraffin and incubated in the cold room overnight in a moist box containing a wet paper towel or at room temperature and humidity for two hours under gentle shaking condition.

3. The plate was emptied and the unoccupied sites are blocked with 200 µl of blocking buffer containing 5-10% of skim-milk in Phosphate buffered saline (PBS) for 1 hr at room temperature.

4. The plate was emptied and washed four times with wash buffer made up of PBS.

5. The primary antibody solution was first diluted in PBS to give a dilution factor of 50000. 100 µl of the diluted antibody was added to each well. The plate was then incubated at room temperature for 45-60 minutes under gentle shaking.

6. The plate was emptied again and washed four times with wash buffer.

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7. The Horse-redish peroxidase enzyme-labeled antibody against antigen was diluted appropriately in PBS. 100  $\mu$ l of this dilution was added to each well and incubated at room temperature for 1 hr.

8. The plate was emptied again and washed six times with 1 $\times$ PBS.

9. To each well 100  $\mu$ l of 1 $\times$ TMB (Tetramethylbenzidine Liquid substrate, Sigma-Aldrich, USA) was added and the plate was left for 10 minutes at room temperature.

To stop the reaction 50  $\mu$ l 1N Sulphuric acid was added to each well and the color development was read spectrophotometrically at 450 nm.

Absorption values at 450 nm in the ELISA, obtained for unmodified nSK and various PEGylated variants of SK, were used for evaluation of the relative levels of their immune-reactivity against SK polyclonal sera raised in rabbit. The ELISA studies showed that conjugation of one PEG group of 20 KDa in any of the three domain of SK reduces its reactivity to well below 20% against SK polyclonal sera. Conjugation of two PEG groups of 20 KDa i.e. one at N- and another at C-terminus of SK reduced their reactivity to well below 10% against SK polyclonal sera. Conjugation of three PEG groups of 20 KDa i.e. one in each domain of SK rendered the reactivity to barely detectable levels. Hence, it is clear that the conjugation of the PEG moiety(ies) to the different regions of SK significantly reduces their reactivity against SK polyclonal sera. In vitro tests showing reduced antibody reactivity established that a reduced induction of immune response occurs once the PEGylated protein is injected into the live animal. Table 33 lists the percent immune reactivity retained in PEGylated SK variants while taking the reactivity of wild type unmodified SK as 100%. The present invention thus discloses PEGylated streptokinase variants with markedly reduced immunoreactivity but intact thrombolytic potency

## Advantages of the Invention

The advantage of the present invention lies in its disclosure of the design of cysteine variants of streptokinase its muteins, species variants and covalently modified forms. Site specific PEG conjugation to the cysteine variants disclosed in this invention imparts various useful therapeutic properties to the streptokinase molecule such as increased proteolytic stability, improved in vivo half life and less immune reactivity. More particularly, the invention relates to production of engineered streptokinase derivatives for use in pharmaceutical compositions for treating circulatory disorders.

TABLE 1

Solvent accessibility values for various amino-acid selected for cysteine substitution.		
SK or variant	Location of cysteine mutation	Accessibility
I 88	88-97 loop of alpha domain	30
S 93	88-97 loop of alpha domain	42
D 95	88-97 loop of alpha domain	132
D 96	88-97 loop of alpha domain	58
D 102	$\beta$ 4 of alpha domain	58
S 105	Preciding the $\beta$ 4'	123
D 120	Region between $\beta$ 5 and $\beta$ 6	65
K 121	Region between $\beta$ 5 and $\beta$ 6 domain	199
D 122	Region between $\beta$ 5 and $\beta$ 6 of alpha domain	101
E 148	Linker region of alpha and beta domain	118

## 32

TABLE 1-continued

Solvent accessibility values for various amino-acid selected for cysteine substitution.		
SK or variant	Location of cysteine mutation	Accessibility
K 156	Linker region of alpha and beta domain	109
D 173	Loop between $\beta$ 1 and $\beta$ 2 of beta domain	145
D 174	Loop between $\beta$ 1 and $\beta$ 2 of beta domain	111
L 179	$\beta$ 2 of beta domain	54
D 181	At the end of $\beta$ 2 of beta domain	36
S 205	$\alpha$ helix ( $\alpha$ 3,4) of beta domain	62
N 255	250 loop of beta domain	41
K 256	250 loop of beta domain	174
K 257	250 loop of beta domain	189
S 258	250 loop of beta domain	79
L 260	250 loop of beta domain	112
K 282	Linker region of beta and gamma domain	154
F 287	Linker region of beta and gamma domain	161
D 303	$\beta$ 1 of gamma domain	134
L 321	Coiled - coil region of gamma domain	47
L 326	Coiled - coil region of gamma domain	12
A 333	Coiled - coil region of gamma domain	24
D 347	$\beta$ 4 of the gamma domain	53
R 372	$\beta$ 7 of the gamma domain	234

TABLE 2

Different constructs of SK its muteins and fusion polypeptides that were used for cysteine mutagenesis	
MOLECULE	MODIFICATION
SEQ ID NO 1	nSK (1-414) ATCC 12449
SEQ ID NO 2	SK (1-383)
SEQ ID NO 3	SK (16-414)
SEQ ID NO 4	SK (16-383)
SEQ ID NO 5	SK (60-414)
SEQ ID NO 6	SK (60-383)
SEQ ID NO 7	SK Asn 90 Ala (1-414)
SEQ ID NO 8	SK Asp 227 Tyr (1-414)
SEQ ID NO 9	SK Asp 238 Ala (1-414)
SEQ ID NO 10	SK Glu 240 Ala (1-414)
SEQ ID NO 11	SK Arg 244 Ala (1-414)
SEQ ID NO 12	SK Lys 246 Ala (1-414)
SEQ ID NO 13	SK Leu 260 Ala (1-414)
SEQ ID NO 14	SK Asp 359 Arg (1-414)
SEQ ID NO 15	SK His, Ser 92, 93 Ala, Ala (1-414)
SEQ ID NO 16	SK Lys, Lys 278, 279 Ala, Ala (1-414)
SEQ ID NO 17	SK Asn 90 del (1-413)
SEQ ID NO 18	SK Asp 227 del (1-413)
SEQ ID NO 19	SK Asp 359 del (1-413)
SEQ ID NO 20	SK (1-406) <i>Streptococcus pyogenes</i> MGAS10270, ABF34818.1
SEQ ID NO 21	SK (1-414) <i>Streptococcus dysgalactiae</i> subsp. <i>Equismitis</i> , AAC60418
SEQ ID NO 22	Fn SK (1-531)
SEQ ID NO 23	SK Fn (1-502)
SEQ ID NO 24	Fn SK Fn (1-619)

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TABLE 3

Cysteine substitution on SK polypeptide (SEQ ID NO 1): 1-414	
MOLECULE	MODIFICATION
SEQ ID NO 25	Gly 49 Cys
SEQ ID NO 26	Ser 57 Cys
SEQ ID NO 27	Ala 64 Cys
SEQ ID NO 28	Ile 88 Cys
SEQ ID NO 29	Ser 93 Cys
SEQ ID NO 30	Asp 95 Cys
SEQ ID NO 31	Asp 96 Cys
SEQ ID NO 32	Asp 102 Cys
SEQ ID NO 33	Asp 105 Cys
SEQ ID NO 34	Asp 120 Cys
SEQ ID NO 35	Lys 121 Cys
SEQ ID NO 36	Asp 122 Cys
SEQ ID NO 37	Glu 148 Cys
SEQ ID NO 38	Lys 156 Cys
SEQ ID NO 39	Asp 173 Cys
SEQ ID NO 40	Asp 174 Cys
SEQ ID NO 41	Leu 179 Cys
SEQ ID NO 42	Asp 181 Cys
SEQ ID NO 43	Ser 205 Cys
SEQ ID NO 44	Ala 251 Cys
SEQ ID NO 45	Ile 254 Cys
SEQ ID NO 46	Asn 255 Cys
SEQ ID NO 47	Lys 256 Cys
SEQ ID NO 48	Lys 257 Cys
SEQ ID NO 49	Ser 258 Cys
SEQ ID NO 50	Leu 260 Cys
SEQ ID NO 51	Glu 281 Cys
SEQ ID NO 52	Lys 282 Cys
SEQ ID NO 53	Phe 287 Cys
SEQ ID NO 54	Asp 303 Cys
SEQ ID NO 55	Leu 321 Cys
SEQ ID NO 56	Leu 326 Cys
SEQ ID NO 57	Ala 333 Cys
SEQ ID NO 58	Asp 347 Cys
SEQ ID NO 59	Asp 360 Cys
SEQ ID NO 60	Arg 372 Cys
SEQ ID NO 61	Ile 88 Cys, Ser 205 Cys
SEQ ID NO 62	Ser 93Cys, Asn 255 Cys
SEQ ID NO 63	Asp 102 Cys, Arg 372 Cys
SEQ ID NO 64	Ser 105 Cys, and Phe 287 Cys
SEQ ID NO 65	Lys 121 Cys, Asp 360 Cys
SEQ ID NO 66	Ile 88 Cys, Ser 205 Cys, Arg 372
SEQ ID NO 67	Ser 93 Cys, Asn 255 Cys, Asp 347 Cys
SEQ ID NO 68	Ser 93 Cys, Asn 255 Cys, Phe 287 Cys
SEQ ID NO 69	Asp 102 Cys, Leu 260 Cys, Arg 372 Cys
SEQ ID NO 70	Ser 105 Cys, Leu 260 Cys, Phe 287 Cys

TABLE 4

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 2): 1-383	
MOLECULE	MODIFICATION
SEQ ID NO 71	Gly 49 Cys
SEQ ID NO 72	Ser 57 Cys
SEQ ID NO 73	Ala 64 Cys
SEQ ID NO 74	Ile 88 Cys
SEQ ID NO 75	Ser 93 Cys
SEQ ID NO 76	Asp 95 Cys
SEQ ID NO 77	Asp 96 Cys
SEQ ID NO 78	Asp 102 Cys
SEQ ID NO 79	Asp 105 Cys
SEQ ID NO 80	Asp 120 Cys
SEQ ID NO 81	Lys 121 Cys
SEQ ID NO 82	Asp 122 Cys
SEQ ID NO 83	Glu 148 Cys
SEQ ID NO 84	Lys 156 Cys
SEQ ID NO 85	Asp 173 Cys
SEQ ID NO 86	Asp 174 Cys
SEQ ID NO 87	Leu 179 Cys
SEQ ID NO 88	Asp 181 Cys
SEQ ID NO 89	Ser 205 Cys

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TABLE 4-continued

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 2): 1-383	
MOLECULE	MODIFICATION
SEQ ID NO 90	Ala 251 Cys
SEQ ID NO 91	Ile 254 Cys
SEQ ID NO 92	Asn 255 Cys
SEQ ID NO 93	Lys 256 Cys
SEQ ID NO 94	Lys 257 Cys
SEQ ID NO 95	Ser 258 Cys
SEQ ID NO 96	Leu 260 Cys
SEQ ID NO 97	Glu 281 Cys
SEQ ID NO 98	Lys 282 Cys
SEQ ID NO 99	Phe 287 Cys
SEQ ID NO 100	Asp 303 Cys
SEQ ID NO 101	Leu 321 Cys
SEQ ID NO 102	Leu 326 Cys
SEQ ID NO 103	Ala 333 Cys
SEQ ID NO 104	Asp 347 Cys
SEQ ID NO 105	Asp 360 Cys
SEQ ID NO 106	Arg 372 Cys
SEQ ID NO 107	Ile 88 Cys, Ser 205 Cys
SEQ ID NO 108	Ser 93Cys, Asn 255 Cys
SEQ ID NO 109	Asp 102 Cys, Arg 372 Cys
SEQ ID NO 110	Ser 105 Cys, and Phe 287 Cys
SEQ ID NO 111	Lys 121 Cys, Asp 360 Cys
SEQ ID NO 112	Ile 88 Cys, Ser 205 Cys, Arg 372
SEQ ID NO 113	Ser 93 Cys, Asn 255 Cys, Asp 347 Cys
SEQ ID NO 114	Ser 93 Cys, Asn 255 Cys, Phe 287 Cys
SEQ ID NO 115	Asp 102 Cys, Leu 260 Cys, Arg 372 Cys
SEQ ID NO 116	Ser 105 Cys, Leu 260 Cys, Phe 287 Cys

TABLE 5

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 3): 16-414	
MOLECULE	MODIFICATION
SEQ ID NO 117	Gly 34 Cys
SEQ ID NO 118	Ser 42 Cys
SEQ ID NO 119	Ala 49 Cys
SEQ ID NO 120	Ile 73 Cys
SEQ ID NO 121	Ser 78 Cys
SEQ ID NO 122	Asp 80 Cys
SEQ ID NO 123	Asp 81 Cys
SEQ ID NO 124	Asp 87 Cys
SEQ ID NO 125	Ser 90 Cys
SEQ ID NO 126	Asp 105 Cys
SEQ ID NO 127	Lys 106 Cys
SEQ ID NO 128	Asp 107 Cys
SEQ ID NO 129	Glu 133 Cys
SEQ ID NO 130	Lys 141 Cys
SEQ ID NO 131	Asp 158 Cys
SEQ ID NO 132	Asp 159 Cys
SEQ ID NO 133	Leu 164 Cys
SEQ ID NO 134	Asp 166 Cys
SEQ ID NO 135	Ser 190 Cys
SEQ ID NO 136	Ala 236 Cys
SEQ ID NO 137	Ile 239 Cys
SEQ ID NO 138	Asn 240 Cys
SEQ ID NO 139	Lys 241 Cys
SEQ ID NO 140	Lys 242 Cys
SEQ ID NO 141	Ser 243 Cys
SEQ ID NO 142	Leu 245 Cys
SEQ ID NO 143	Glu 266 Cys
SEQ ID NO 144	Lys 267 Cys
SEQ ID NO 145	Phe 272 Cys
SEQ ID NO 146	Asp 288 Cys
SEQ ID NO 147	Leu 306 Cys
SEQ ID NO 148	Leu 311 Cys
SEQ ID NO 149	Ala 318 Cys
SEQ ID NO 150	Asp 332 Cys
SEQ ID NO 151	Asp 345 Cys
SEQ ID NO 152	Arg 357 Cys
SEQ ID NO 153	Ile 73 Cys, Ser 190 Cys

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TABLE 5-continued

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 3): 16-414	
MOLECULE	MODIFICATION
SEQ ID NO 154	Ser 78 Cys, Asn 240 Cys
SEQ ID NO 155	Asp 87 Cys, Arg 357 Cys
SEQ ID NO 156	Ser 90 Cys, and Phe 272 Cys
SEQ ID NO 157	Lys 106 Cys, Asp 345 Cys
SEQ ID NO 158	Ile 73 Cys, Ser 190 Cys, Arg 357 Cys
SEQ ID NO 159	Ser 78 Cys, Asn 240 Cys, Asp 332 Cys
SEQ ID NO 160	Ser 78 Cys, Asn 240 Cys, Phe 272 Cys
SEQ ID NO 161	Asp 87 Cys, Leu 245 Cys, Arg 357 Cys
SEQ ID NO 162	Ser 90 Cys, Leu 245 Cys, Phe 272 Cys

TABLE 6

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 4): 16-383	
MOLECULE	MODIFICATION
SEQ ID NO 163	Gly 34 Cys
SEQ ID NO 164	Ser 42 Cys
SEQ ID NO 165	Ala 49 Cys
SEQ ID NO 166	Ile 73 Cys
SEQ ID NO 167	Ser 78 Cys
SEQ ID NO 168	Asp 80 Cys
SEQ ID NO 169	Asp 81 Cys
SEQ ID NO 170	Asp 87 Cys
SEQ ID NO 171	Ser 90 Cys
SEQ ID NO 172	Asp 105 Cys
SEQ ID NO 173	Lys 106 Cys
SEQ ID NO 174	Asp 107 Cys
SEQ ID NO 175	Glu 133 Cys
SEQ ID NO 176	Lys 141 Cys
SEQ ID NO 177	Asp 158 Cys
SEQ ID NO 178	Asp 159 Cys
SEQ ID NO 179	Leu 164 Cys
SEQ ID NO 180	Asp 166 Cys
SEQ ID NO 181	Ser 190 Cys
SEQ ID NO 182	Ala 236 Cys
SEQ ID NO 183	Ile 239 Cys
SEQ ID NO 184	Asn 240 Cys
SEQ ID NO 185	Lys 241 Cys
SEQ ID NO 186	Lys 242 Cys
SEQ ID NO 187	Ser 243 Cys
SEQ ID NO 188	Leu 245 Cys
SEQ ID NO 189	Glu 266 Cys
SEQ ID NO 190	Lys 267 Cys
SEQ ID NO 191	Phe 272 Cys
SEQ ID NO 192	Asp 288 Cys
SEQ ID NO 193	Leu 306 Cys
SEQ ID NO 194	Leu 311 Cys
SEQ ID NO 195	Ala 318 Cys
SEQ ID NO 196	Asp 332 Cys
SEQ ID NO 197	Asp 345 Cys
SEQ ID NO 198	Arg 357 Cys
SEQ ID NO 199	Ile 73 Cys, Ser 190 Cys
SEQ ID NO 200	Ser 78 Cys, Asn 240 Cys
SEQ ID NO 201	Asp 87 Cys, Arg 357 Cys
SEQ ID NO 202	Ser 90 Cys, and Phe 272 Cys
SEQ ID NO 203	Lys 106 Cys, Asp 345 Cys
SEQ ID NO 204	Ile 73 Cys, Ser 190 Cys, Arg 357 Cys
SEQ ID NO 205	Ser 78 Cys, Asn 240 Cys, Asp 332 Cys
SEQ ID NO 206	Ser 78 Cys, Asn 240 Cys, Phe 272 Cys
SEQ ID NO 207	Asp 87 Cys, Leu 245 Cys, Arg 357 Cys
SEQ ID NO 208	Ser 90 Cys, Leu 245 Cys, Phe 272 Cys

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TABLE 7

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 5): 60-414	
MOLECULE	MODIFICATION
SEQ ID NO 209	Ala 64 Cys
SEQ ID NO 210	Ile 88 Cys
SEQ ID NO 211	Ser 44 Cys
SEQ ID NO 212	Asp 46 Cys
SEQ ID NO 213	Asp 47 Cys
SEQ ID NO 214	Asp 53 Cys
SEQ ID NO 215	Ser 56 Cys
SEQ ID NO 216	Asp 71 Cys
SEQ ID NO 217	Lys 72 Cys
SEQ ID NO 218	Asp 73 Cys
SEQ ID NO 219	Glu 99 Cys
SEQ ID NO 220	Lys 107 Cys
SEQ ID NO 221	Asp 124 Cys
SEQ ID NO 222	Asp 125 Cys
SEQ ID NO 223	Leu 130 Cys
SEQ ID NO 224	Asp 132 Cys
SEQ ID NO 225	Ser 156 Cys
SEQ ID NO 226	Ala 202 Cys
SEQ ID NO 227	Ile 205 Cys
SEQ ID NO 228	Asn 206 Cys
SEQ ID NO 229	Lys 207 Cys
SEQ ID NO 230	Lys 208 Cys
SEQ ID NO 231	Ser 209 Cys
SEQ ID NO 232	Leu 211 Cys
SEQ ID NO 233	Glu 232 Cys
SEQ ID NO 234	Lys 233 Cys
SEQ ID NO 235	Phe 238 Cys
SEQ ID NO 236	Asp 254 Cys
SEQ ID NO 237	Leu 272 Cys
SEQ ID NO 238	Leu 277 Cys
SEQ ID NO 239	Ala 284 Cys
SEQ ID NO 240	Asp 298 Cys
SEQ ID NO 241	Asp 311 Cys
SEQ ID NO 242	Arg 323 Cys
SEQ ID NO 243	Ile 39 Cys, Ser 156 Cys
SEQ ID NO 244	Ser 44 Cys, Asn 206 Cys
SEQ ID NO 245	Asp 53 Cys, Arg 323 Cys
SEQ ID NO 246	Ser 56 Cys, and Phe 238 Cys
SEQ ID NO 247	Lys 72 Cys, Asp 311 Cys
SEQ ID NO 248	Ile 39 Cys, Ser 156 Cys, Arg 323 Cys
SEQ ID NO 249	Ser 44 Cys, Asn 255 Cys, Asp 347 Cys
SEQ ID NO 250	Ser 93 Cys, Asn 255 Cys, Phe 287 Cys
SEQ ID NO 251	Asp 53 Cys, Leu 211 Cys, Arg 323 Cys
SEQ ID NO 252	Ser 56 Cys, Leu 211 Cys, Phe 238 Cys

TABLE 8

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 6): 60-383	
MOLECULE	MODIFICATION
SEQ ID NO 253	Ala 5 Cys
SEQ ID NO 254	Ile 29 Cys
SEQ ID NO 255	Ser 34 Cys
SEQ ID NO 256	Asp 36 Cys
SEQ ID NO 257	Asp 37 Cys
SEQ ID NO 258	Asp 43 Cys
SEQ ID NO 259	Ser 46 Cys
SEQ ID NO 260	Asp 61 Cys
SEQ ID NO 261	Lys 62 Cys
SEQ ID NO 262	Asp 63 Cys
SEQ ID NO 263	Glu 89 Cys
SEQ ID NO 264	Lys 97 Cys
SEQ ID NO 265	Asp 114 Cys
SEQ ID NO 266	Asp 115 Cys
SEQ ID NO 267	Leu 120 Cys
SEQ ID NO 268	Asp 122 Cys
SEQ ID NO 269	Ser 146 Cys
SEQ ID NO 270	Ala 192 Cys
SEQ ID NO 271	Ile 195 Cys
SEQ ID NO 272	Asn 196 Cys

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TABLE 8-continued

Single Cysteine substitution on truncated SK polypeptide (SEQ ID NO 6): 60-383	
MOLECULE	MODIFICATION
SEQ ID NO 273	Lys 197 Cys
SEQ ID NO 274	Lys 198 Cys
SEQ ID NO 275	Ser 199 Cys
SEQ ID NO 276	Leu 201 Cys
SEQ ID NO 277	Glu 222 Cys
SEQ ID NO 278	Lys 223 Cys
SEQ ID NO 279	Phe 287228 Cys
SEQ ID NO 280	Asp 244 Cys
SEQ ID NO 281	Leu 262 Cys
SEQ ID NO 282	Leu 267 Cys
SEQ ID NO 283	Ala 274 Cys
SEQ ID NO 284	Asp 188 Cys
SEQ ID NO 285	Asp 301 Cys
SEQ ID NO 286	Arg 313 Cys
SEQ ID NO 287	Ile 29 Cys, Ser 146 Cys
SEQ ID NO 288	Ser 34 Cys, Asn 196 Cys
SEQ ID NO 289	Asp 43 Cys, Arg 313 Cys
SEQ ID NO 290	Ser 46 Cys, and Phe 228 Cys
SEQ ID NO 291	Lys 62 Cys, Asp 301 Cys
SEQ ID NO 292	Ile 29 Cys, Ser 146 Cys, Arg 313 Cys
SEQ ID NO 293	Ser 34 Cys, Asn 196 Cys, Asp 288 Cys
SEQ ID NO 294	Ser 34 Cys, Asn 196 Cys, Phe 228 Cys
SEQ ID NO 295	Asp 43 Cys, Leu 201 Cys, Arg 313 Cys
SEQ ID NO 296	Ser 46 Cys, Leu 201 Cys, Phe 228 Cys

TABLE 9

Cysteine variants of SK 1-414 mutein, Asn 90 Ala: (SEQ ID NO 7)	
MOLECULE	MODIFICATION
SEQ ID NO 297	Asp 102 Cys
SEQ ID NO 298	Leu 260 Cys
SEQ ID NO 299	Asp 347 Cys

TABLE 10

Cysteine variants of SK (1-414) mutein, Asp 227 Tyr: (SEQ ID NO 8)	
MOLECULE	MODIFICATION
SEQ ID NO 300	Asp 102 Cys
SEQ ID NO 301	Leu 260 Cys
SEQ ID NO 302	Asp 347 Cys

TABLE 11

Cysteine variants of SK (1-414) mutein, Asp 238 Ala: (SEQ ID NO 9)	
MOLECULE	MODIFICATION
SEQ ID NO 303	Asp 102 Cys
SEQ ID NO 304	Leu 260 Cys
SEQ ID NO 305	Asp 347 Cys

TABLE 12

Cysteine variants of SK (1-414) mutein, Glu 240 Ala: (SEQ ID NO 10)	
MOLECULE	MODIFICATION
SEQ ID NO 306	Asp 102 Cys
SEQ ID NO 307	Leu 260 Cys
SEQ ID NO 308	Asp 347 Cys

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TABLE 13

Cysteine variants of SK (1-414) mutein, Arg 244 Ala: (SEQ ID NO 11)	
MOLECULE	MODIFICATION
SEQ ID NO 309	Asp 102 Cys
SEQ ID NO 310	Leu 260 Cys
SEQ ID NO 311	Asp 347 Cys

TABLE 14

Cysteine variants of SK (1-414) mutein, Lys 246 Ala: (SEQ ID NO 12)	
MOLECULE	MODIFICATION
SEQ ID NO 312	Asp 102 Cys
SEQ ID NO 313	Leu 260 Cys
SEQ ID NO 314	Asp 347 Cys

TABLE 15

Cysteine variants of SK (1-414) mutein, Leu 260 Ala: (SEQ ID NO 13)	
MOLECULE	MODIFICATION
SEQ ID NO 315	Asp 102 Cys
SEQ ID NO 316	Asn 255 Cys
SEQ ID NO 317	Asp 347 Cys

TABLE 16

Cysteine variants of SK (1-414) mutein, Asp 359 Arg: (SEQ ID NO 14)	
MOLECULE	MODIFICATION
SEQ ID NO 318	Asp 102 Cys
SEQ ID NO 319	Leu 260 Cys
SEQ ID NO 320	Asp 347 Cys

TABLE 17

Cysteine variants of SK (1-414) mutein, His, Ser, 92, 93 Ala, Ala: (SEQ ID NO 15)	
MOLECULE	MODIFICATION
SEQ ID NO 321	Asp 102 Cys
SEQ ID NO 322	Leu 260 Cys
SEQ ID NO 323	Asp 347 Cys

TABLE 18

Cysteine variants of SK (1-414) mutein, Lys, Lys 278, 279 Ala, Ala: (SEQ ID NO 16)	
MOLECULE	MODIFICATION
SEQ ID NO 324	Asp 102 Cys
SEQ ID NO 325	Leu 260 Cys
SEQ ID NO 326	Asp 347 Cys

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TABLE 19

Cysteine variants of SK (1-414) mutein, Asn 90 del: (SEQ ID NO 17)	
MOLECULE	MODIFICATION
SEQ ID NO 327	Asp 102 Cys
SEQ ID NO 328	Leu 260 Cys
SEQ ID NO 329	Asp 347 Cys

TABLE 20

Cysteine variants of SK (1-414) mutein, Asp 227 del: (SEQ ID NO 18)	
MOLECULE	MODIFICATION
SEQ ID NO 330	Asp 102 Cys
SEQ ID NO 331	Leu 260 Cys
SEQ ID NO 332	Asp 347 Cys

TABLE 21

Cysteine variants of SK (1-414) mutein, Asp 359 del: (SEQ ID NO 19)	
MOLECULE	MODIFICATION
SEQ ID NO 333	Asp 102 Cys
SEQ ID NO 334	Leu 260 Cys
SEQ ID NO 335	Asp 347 Cys

TABLE 22

Cysteine variants of <i>Streptococcus pyogenes</i> MGAS10270 (SEQ ID NO 20)	
MOLECULE	MODIFICATION
SEQ ID NO 336	Ile 80 Cys
SEQ ID NO 337	Ser 85 Cys
SEQ ID NO 338	Asp 94 Cys
SEQ ID NO 339	Ile 246 Cys
SEQ ID NO 340	Asp 339 Cys
SEQ ID NO 341	Arg 364 Cys

TABLE 23

Cysteine variants of <i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i> (SEQ ID NO 21)	
MOLECULE	MODIFICATION
SEQ ID NO 342	Ile 88 Cys
SEQ ID NO 343	Ser 93 Cys
SEQ ID NO 344	Asp 102 Cys
SEQ ID NO 345	Leu 260 Cys
SEQ ID NO 346	Asp 347 Cys
SEQ ID NO 347	Arg 372 Cys

TABLE 24

Cysteine variants of polypeptide where fibrin domain is fused to N-terminus of SK (SEQ ID NO 22): 1-531	
MOLECULE	MODIFICATION
SEQ ID NO 348	His 16 Cys
SEQ ID NO 349	Ala 17 Cys
SEQ ID NO 350	Asp 62 Cys
SEQ ID NO 351	Gly 80 Cys
SEQ ID NO 352	Gly 166 Cys

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TABLE 24-continued

Cysteine variants of polypeptide where fibrin domain is fused to N-terminus of SK (SEQ ID NO 22): 1-531	
MOLECULE	MODIFICATION
SEQ ID NO 353	Ser 174 Cys
SEQ ID NO 354	Ala 181 Cys
SEQ ID NO 355	Ile 205 Cys
SEQ ID NO 356	Ser 210 Cys
SEQ ID NO 357	Asp 212 Cys
SEQ ID NO 358	Asp 213 Cys
SEQ ID NO 359	Asp 219 Cys
SEQ ID NO 360	Ser 222 Cys
SEQ ID NO 361	Asp 237 Cys
SEQ ID NO 362	Lys 238 Cys
SEQ ID NO 363	Asp 239 Cys
SEQ ID NO 364	Glu 265 Cys
SEQ ID NO 365	Lys 273 Cys
SEQ ID NO 366	Asp 290 Cys
SEQ ID NO 367	Asp 291 Cys
SEQ ID NO 368	Leu 296 Cys
SEQ ID NO 369	Asp 298 Cys
SEQ ID NO 370	Ser 322 Cys
SEQ ID NO 371	Ile 371 Cys
SEQ ID NO 372	Asn 372 Cys
SEQ ID NO 373	Lys 373 Cys
SEQ ID NO 374	Lys 374 Cys
SEQ ID NO 375	Ser 375 Cys
SEQ ID NO 376	Leu 377 Cys
SEQ ID NO 377	Glu 398 Cys
SEQ ID NO 378	Lys 399 Cys
SEQ ID NO 379	Phe 404 Cys
SEQ ID NO 380	Asp 420 Cys
SEQ ID NO 381	Leu 438 Cys
SEQ ID NO 382	Leu 443 Cys
SEQ ID NO 383	Ala 450 Cys
SEQ ID NO 384	Asp 464 Cys
SEQ ID NO 385	Asp 477 Cys
SEQ ID NO 386	Arg 489 Cys
SEQ ID NO 387	His 16 Cys, Ile 205 Cys
SEQ ID NO 388	His 16 Cys, Ser 322 Cys
SEQ ID NO 389	His 16 Cys, Leu 377 Cys
SEQ ID NO 390	His 16 Cys, Arg 489 Cys

TABLE 25

Cysteine variants of polypeptide where fibrin domain is fused to C-terminal of SK (SEQ ID NO 23): 1-502	
MOLECULE	MODIFICATION
SEQ ID NO 391	Gly 49 Cys
SEQ ID NO 392	Ser 57 Cys
SEQ ID NO 393	Ala 64 Cys
SEQ ID NO 394	Ile 88 Cys
SEQ ID NO 395	Ser 93 Cys
SEQ ID NO 396	Asp 95 Cys
SEQ ID NO 397	Asp 96 Cys
SEQ ID NO 398	Asp 102 Cys
SEQ ID NO 399	Asp 105 Cys
SEQ ID NO 400	Asp 120 Cys
SEQ ID NO 401	Lys 121 Cys
SEQ ID NO 402	Asp 122 Cys
SEQ ID NO 403	Glu 148 Cys
SEQ ID NO 404	Lys 156 Cys
SEQ ID NO 405	Asp 173 Cys
SEQ ID NO 406	Asp 174 Cys
SEQ ID NO 407	Leu 179 Cys
SEQ ID NO 408	Asp 181 Cys
SEQ ID NO 409	Ser 205 Cys
SEQ ID NO 410	Ala 251 Cys
SEQ ID NO 411	Ile 254 Cys
SEQ ID NO 412	Asn 255 Cys
SEQ ID NO 413	Lys 256 Cys
SEQ ID NO 414	Lys 257 Cys
SEQ ID NO 415	Ser 258 Cys
SEQ ID NO 416	Leu 260 Cys



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TABLE 25-continued

Cysteine variants of polypeptide where fibrin domain is fused to C-terminal of SK (SEQ ID NO 23): 1-502	
MOLECULE	MODIFICATION
SEQ ID NO 417	Glu 281 Cys
SEQ ID NO 418	Lys 282 Cys
SEQ ID NO 419	Phe 287 Cys
SEQ ID NO 420	Asp 303 Cys
SEQ ID NO 421	Leu 321 Cys
SEQ ID NO 422	Leu 326 Cys
SEQ ID NO 423	Ala 333 Cys
SEQ ID NO 424	Asp 347 Cys
SEQ ID NO 425	Asp 360 Cys
SEQ ID NO 426	Arg 372 Cys
SEQ ID NO 427	His 401 Cys
SEQ ID NO 428	Ala 402 Cys
SEQ ID NO 429	Asp 447 Cys
SEQ ID NO 430	Gly 465 Cys
SEQ ID NO 431	Ile 88 Cys, His 401 Cys
SEQ ID NO 432	Ser 205 Cys, His 401 Cys
SEQ ID NO 433	Leu 260 Cys, His 401 Cys
SEQ ID NO 434	Arg 372 Cys, His 401 Cys

TABLE 26

Cysteine variants of polypeptide where fibrin domain is fused to both N and C-terminus of SK (SEQ ID NO 24): 1-619	
MOLECULE	MODIFICATION
SEQ ID NO 435	His 16 Cys
SEQ ID NO 436	Ala 17 Cys
SEQ ID NO 437	Asp 62 Cys
SEQ ID NO 438	Gly 80 Cys
SEQ ID NO 439	Gly 166 Cys
SEQ ID NO 440	Ser 157 Cys
SEQ ID NO 441	Ala 181 Cys
SEQ ID NO 442	Ile 205 Cys
SEQ ID NO 443	Ser 210 Cys
SEQ ID NO 444	Asp 212 Cys
SEQ ID NO 445	Asp 213 Cys
SEQ ID NO 446	Asp 219 Cys
SEQ ID NO 447	Asp 212 Cys
SEQ ID NO 448	Asp 237 Cys
SEQ ID NO 449	Lys 238 Cys
SEQ ID NO 450	Asp 239 Cys
SEQ ID NO 451	Glu 265 Cys
SEQ ID NO 452	Lys 273 Cys
SEQ ID NO 453	Asp 290 Cys
SEQ ID NO 454	Asp 291 Cys
SEQ ID NO 455	Leu 296 Cys
SEQ ID NO 456	Asp 298 Cys
SEQ ID NO 457	Ser 322 Cys
SEQ ID NO 458	Ile 371 Cys
SEQ ID NO 459	Asn 372 Cys
SEQ ID NO 460	Lys 373 Cys
SEQ ID NO 461	Lys 374 Cys
SEQ ID NO 462	Ser 375 Cys
SEQ ID NO 463	Leu 377 Cys
SEQ ID NO 464	Glu 398 Cys
SEQ ID NO 465	Lys 399 Cys
SEQ ID NO 466	Phe 404 Cys
SEQ ID NO 467	Asp 420 Cys
SEQ ID NO 468	Leu 438 Cys
SEQ ID NO 469	Leu 443 Cys
SEQ ID NO 470	Ala 450 Cys
SEQ ID NO 471	Asp 464 Cys
SEQ ID NO 472	Asp 477 Cys
SEQ ID NO 473	Arg 489 Cys
SEQ ID NO 474	His 518 Cys
SEQ ID NO 475	Ala 519 Cys
SEQ ID NO 476	Asp 564 Cys
SEQ ID NO 477	Gly 582 Cys
SEQ ID NO 478	His 16 Cys, Leu 377 Cys
SEQ ID NO 479	His 16 Cys, Ser 322 Cys,
SEQ ID NO 480	His 16 Cys, His 518 Cys

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TABLE 26-continued

Cysteine variants of polypeptide where fibrin domain is fused to both N and C-terminus of SK (SEQ ID NO 24): 1-619	
MOLECULE	MODIFICATION
SEQ ID NO 481	Ala 17 Cys, Ala 519 Cys
SEQ ID NO 482	Asp 62 Cys, Asp 564 Cys
SEQ ID NO 483	Gly 80 Cys, Gly 582 Cys
SEQ ID NO 484	His 16 Cys, Leu 377 Cys, Arg 489 Cys
SEQ ID NO 485	His 16 Cys, Leu 377 Cys, His 518 Cys

TABLE 27

Cysteine insertion mutants of SK	
MOLECULE	MODIFICATION
SEQ ID NO 486	Cys between Ile 88 and Ala 89 of SK
SEQ ID NO 487	Cys between Lys 256 and Lys 257 of SK
SEQ ID NO 488	Cys between Asp 347 and Tyr 348 of SK

TABLE 28

Cysteine variants of SK where Cys is placed at the termini of SK or its functional fragment with or without a peptide extension	
MOLECULE	MODIFICATION
SEQ ID NO 489	Cys at N - 1 position in SK
SEQ ID NO 490	Cys at C + 1 position in SK
SEQ ID NO 491	Cys at N - 1 and at C + 1 position in SK
SEQ ID NO 492	Cys at C + 1 position of SEQ ID (1-383, G3 of SK)
SEQ ID NO 493	Cys at N - 1 and at C + 1 position in SK (1-383, G3)
SEQ ID NO 494	Cys in N-terminally His tagged SK
SEQ ID NO 495	Cys at C-terminally His tagged SK
SEQ ID NO 496	Cys with Additional 20 aa tag of pET 15b before Ile

TABLE 29

Steady-state kinetic parameters for some representative PEGylated cysteine variants of PEGylated streptokinases. Activity of native SK is being taken as 100% to compare the activities of variants			
SEQ ID NO	Activator protein	% Plasminogen activation	Km (μM)
SEQ ID NO 1	nSK	100	0.4 ± 0.1
SEQ ID NO 489	N-Cys	88 ± 5	0.4 ± 0.05
SEQ ID NO 30	SK D95C	96 ± 6	0.5 ± 0.05
SEQ ID NO 31	SK D96C	98 ± 4	0.5 ± 0.05
SEQ ID NO 32	SK D102C	74 ± 4	0.5 ± 0.1
SEQ ID NO 33	SK S105C	76 ± 5	0.5 ± 0.12
SEQ ID NO 35	SK K121C	95 ± 5	0.4 ± 0.11
SEQ ID NO 39	SK D173C	72 ± 4	0.4 ± 0.11
SEQ ID NO 41	SK L179C	22 ± 3	0.5 ± 0.12
SEQ ID NO 46	SK N255C	98 ± 3	0.4 ± 0.13
SEQ ID NO 48	SK N257C	92 ± 4	0.4 ± 0.10
SEQ ID NO 49	L258C	100 ± 6	0.5 ± 0.11
SEQ ID NO 50	L260C	100 ± 6	0.6 ± 0.12
SEQ ID NO 492	C-383 Cys	92 ± 4	0.4 ± 0.05
SEQ ID NO 490	C-Cys	95 ± 5	0.4 ± 0.06

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TABLE 30

Steady state kinetic parameters for HPG activation by PEGylated Cysteine variants of fibrin domain fusion forms of SK\*

PEG Variants of SEQ ID NO	Molecule	Lag (min)	Km ( $\mu$ M)	% Plasminogen# Activation
SEQ ID NO 22	SK Fn	10	0.25-0.6	80-100
SEQ ID NO 23	Fn SK	08	0.24-0.63	60-100
SEQ ID NO 24	Fn SK Fn	18	0.48 $\pm$ 1.0	60-80

\*The parameters were calculated from the linear phases of the reaction progress curves after abolishment of the lag phase.

#Expressed relative to the activity of native SK from *streptococcus* sp. (ATCC 12,499), taken as 100%

TABLE 31

Steady-state kinetics parameters for HPG activation by SK and the bi-pegylated SK variants. Activity of native SK is being taken as 100% to compare the activities of variants

SEQ ID NO	Molecule	Lag (min)	Km ( $\mu$ M)	% Plasminogen Activation	% Plasminogen Activation*
SEQ ID NO 1	nSK	1	0.45 $\pm$ 0.02	100	100
SEQ ID NO 491	bi-pegylated NC 1-414	14	0.42 $\pm$ 0.03	<5	88 $\pm$ 4
SEQ ID NO 493	bi-pegylated NC 1-383	12	0.48 $\pm$ 0.02	<5	92 $\pm$ 6

\*plasminogen activation ability when the SK or variants were pre-complexed with equimolar plasmin to make SK.PN enzyme complex.

TABLE 32

In vivo half-life in mice for different PEGylated variants of SK and Clot-specific SK

SEQ ID NO	Molecule	Site of mutation	Half life (t <sub>1/2</sub> )
SEQ ID NO 1	nSK	—	<15 min
SEQ ID NO 489	N-cys	Just after start codon methionine	>3 hrs
SEQ ID NO 30	D 95 C	88-97 loop of alpha domain	>4 hrs
SEQ ID NO 31	D 96 C	88-97 loop of alpha domain	>4 hrs
SEQ ID NO 48	K 257 C	250 loop of beta domain	>2 hrs
SEQ ID NO 49	S 258 C	250 loop of beta domain	>2 hrs
SEQ ID NO 50	L 260 C	250 loop of beta domain	>2 hrs
SEQ ID NO 487	KK 256, 257 KCK	Inserted between Lys 256 and Lys 257 of the 250 loop of beta domain	>2 hrs
SEQ ID NO 55	L 321 C	Coiled - coil region of gamma domain	>1 hr
SEQ ID NO 58	D 347 C	$\beta$ 4 of the gamma domain	>1 hr
SEQ ID NO 492	C-383 Cys	C-terminal truncation at 383 position and cysteine placed after three glycine residues	>1 hr
SEQ ID NO 490	C-cys	Cysteine after the C-terminal amino-acid	>1 hr
SEQ ID NO 491	bi-pegylated NC 1-414	cysteine both at N and C-terminus	>6 hrs
SEQ ID NO 493	bi-pegylated NC 1-383	cysteine at N-terminus and at the C-terminus where cysteine is placed after three Gly following truncation at 383	>6 hrs

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TABLE 33

Immune reactivity of PEGylated cysteine variants of Streptokinase

SEQ ID NO	PEG variant	Immune reactivity*
SEQ ID NO 30	D95C	15 $\pm$ 2
SEQ ID NO 31	D96C	14 $\pm$ 2.5
SEQ ID NO 37	E148C	16 $\pm$ 5
SEQ ID NO 38	K156C	22 $\pm$ 4
SEQ ID NO 41	L179C	17 $\pm$ 5
SEQ ID NO 43	S205C	19 $\pm$ 3
SEQ ID NO 50	L260C	17 $\pm$ 2
SEQ ID NO 490	C-Cys	19 $\pm$ 2

TABLE 33-continued

SEQ ID NO	PEG variant	Immune reactivity*
SEQ ID NO 491	N Cys C Cys	7 $\pm$ 1
SEQ ID NO 62	S93C, N255C	2 $\pm$ .02
SEQ ID NO 63	D102C, R372C	2.5 $\pm$ .02
SEQ ID NO 61	I88C, S205C	2 $\pm$ .05
SEQ ID NO 67	S93C, N255C, D347C	<1
SEQ ID NO 68	S93C, N255C, F287C	<1

\*Immune reactivity were measured against anti SK antibodies raised in rabbit. Values are presented in % reactivity retained while taking wild type reactivity as 100%.

TABLE 34

Highly buried residues of Streptokinase unsuitable for substitution and further modification

Residue No. in SEQ ID 1	Amino-acid	Surface accessibility
18	L	0
19	V	0
20	V	1
22	V	8
24	G	5
42	L	0
79	L	9
83	I	2
87	L	4
97	Y	9
124	S	0
127	L	0
133	Q	7
135	L	0
137	F	4
141	V	3
143	V	2
155	A	4
158	V	0
160	V	1
162	Y	1

TABLE 34-continued

Highly buried residues of Streptokinase unsuitable for substitution and further modification		
Residue No. in SEQ ID 1	Amino-acid	Surface accessibility
169	L	8
196	S	0
203	A	0
207	L	1
222	S	4
224	V	1
226	H	2
245	V	7
266	N	4
268	D	0
270	I	5
272	E	1
274	Y	4
276	V	3
295	F	1
297	I	0
299	Y	0
313	L	3
315	T	2
324	R	3
331	D	0
335	L	1
337	Y	2
340	L	1
342	A	6
365	I	1
367	V	0
369	M	0

The following articles and disclosures are incorporated by reference herein.

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- A251, I254, N255, K256, K257, S258, L260, E281, K282, F287, D303, L321, L326, A333, D347, D360, R372, H401, A402, D447, and G465, and wherein said mutant has biological activity as measured by a standard assay.
6. The mutant streptokinase as claimed in claim 1, wherein the substituted cysteine residue is modified with a cysteine-reactive moiety.
7. The mutant streptokinase as claimed in claim 6, wherein the substituted cysteine residue is modified with polyethylene glycol.

## SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US09115210B2>). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. A purified mutant streptokinase polypeptide having a cysteine residue substituted for at least one amino acid selected from the group consisting of: H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477, R489, H518, A519, D564, and G582, relative to the sequence of SEQ ID NO: 24, and wherein said mutant has biological activity as measured by a standard assay.

2. The mutant streptokinase as claimed in claim 1, wherein said mutant streptokinase is a covalently modified hybrid polypeptide comprising the sequence of one of SEQ ID NO: 22-24 and further comprising

fibrin binding domains 4 and 5, or fibrin binding domains 1 and 2, of human fibronectin.

3. The mutant streptokinase as claimed in claim 1, wherein the mutant further comprises an N and/or C-terminus extension of amino acids.

4. A mutant streptokinase having the amino acid sequence of SEQ ID NO: 22, wherein a cysteine residue is substituted for at least an amino acid selected from the group consisting of H16, A17, D62, G80, G166, S157, A181, I205, S210, D212, D213, D219, D222, D237, K238, D239, E265, K273, D290, D291, L296, D298, S322, I1371, N372, K373, K374, S375, L377, E398, K399, F404, D420, L438, L443, A450, D464, D477, and R489, and wherein said mutant has biological activity as measured by a standard assay.

5. A mutant streptokinase having the amino acid sequence of SEQ ID NO: 23, wherein a cysteine residue is substituted for at least an amino acid selected from the group consisting of: G49, S57, A64, I88, S93, D95, D96, D102, S105, D120, K121, D122, E148, K156, D173, D174, L179, D181, S205,

8. The mutant streptokinase as claimed in claim 7, wherein said PEG molecule is a linear or branch polymer of molecular size ranging from 5000 daltons-40,000 daltons.

9. The mutant streptokinase as claimed in claim 8, wherein said mutant has increased proteolytic stability as compared to their original unmodified counterparts.

10. The mutant streptokinase as claimed in claim 7, wherein said mutant has decreased antigenicity and in vivo immunogenicity when compared to their original unmodified counterparts.

11. The mutant streptokinase as claimed in claim 7, wherein said mutant has slow renal clearance and increased in vivo half life as compared to their original unmodified counterparts.

12. A pharmaceutical composition comprising the mutant streptokinase of claim 1, optionally along with pharmaceutically acceptable excipient(s).

13. The pharmaceutical composition as claimed in claim 12, for treating disease or disorder selected from the group consisting of myocardial infarction, vascular thromboses, pulmonary embolism, stroke a vascular event, angina, pulmonary embolism, transient ischemic attack, deep vein thrombosis, thrombotic re-occlusion subsequent to a coronary intervention procedure, peripheral vascular thrombosis, heart surgery or vascular surgery, heart failure, Syndrome X and a disorder in which a narrowing of at least one coronary artery occurs.

14. A polypeptide comprised of the amino acid sequence as set forth in SEQ ID NO. 24.

15. A pharmaceutical composition comprising the polypeptide of claim 14 in association with pharmaceutically acceptable excipients.